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THE BIOCHEMISTRY OF OLIGOMYCIN RESISTANCE IN  
MUTANTS OF THE YEAST SACCHAROMYCES CEREVISIAE

A thesis submitted in partial fulfilment of  
the requirements for the award of the degree of  
Doctor of Philosophy of the University of Warwick.

by

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March 1973

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### ACKNOWLEDGEMENTS

I would like to express my thanks to the many people who have helped and encouraged this research. In particular I would like to thank Prof. R. B. Beechey, Dr. D. E. Griffiths and Dr. K. J. Cattell. I also thank the S.R.C. for a CAPS post-graduate award and Shell Research Ltd., for so generously providing research facilities during the course of this work. Also R-M.A. without whose patience and understanding this would not have been possible.

## CONTENTS

	<u>Page</u>
<u>CHAPTER 1.</u>	
<u>INTRODUCTION.</u>	1.1.
 <u>CHAPTER 2.</u>	
<u>MATERIALS AND METHODS.</u>	
2.1. Materials.	2.1.
2.2. Growth of Yeast.	2.1.
2.3. Preparation of SMP from cultured cells.	2.1.
2.4. Preparation of rat liver mitochondria.	2.2.
2.5. Preparation of beef heart mitochondria.	2.3.
2.6. Lipid extraction by chloroform-methanol.	2.3.
2.7. Thin layer chromatography.	2.3.
2.8. Radioactivity measurements.	2.4.
2.9. Polyacrylamide gel electrophoresis.	2.5.
2.10. ATPase measurements.	2.6.
2.11. Oxygen electrode measurements.	2.7.
2.12. Pyridine nucleotide transhydrogenase measurements.	2.8.
2.13. Protein measurements.	2.8.
2.14. Phosphate assay.	2.8.
2.15. Extraction of aurovertin.	2.8.
2.16. Ether extraction of yeast SMP.	2.9.
2.17. Saponification and GLC techniques.	2.9.
 <u>CHAPTER 3.</u>	
<u>A COMPARATIVE STUDY OF SMP FROM D22 WT AND VARIOUS OLIGOMYCIN RESISTANT STRAINS.</u>	
3.1. <u>INTRODUCTION.</u>	3.1.
Results	3.2.
3.2. Yeast culture and growth.	3.2.
3.3. Oligomycin sensitivity of the SMP ATPase.	3.2.
3.4. Polyacrylamide gel electrophoresis of the SMP.	3.4.
3.5. Lipid analysis of the SMP.	3.5.
3.6. Fluorescence studies with yeast SMP.	3.6.
3.7. Discussion and conclusions.	3.6.
 <u>CHAPTER 4.</u>	
<u>A STUDY OF THE EFFECTS OF AUROVERTIN AND OLIGOMYCIN ON <sup>32</sup>Pi INCORPORATION INTO MITOCHONDRIA.</u>	
<u>INTRODUCTION.</u>	4.1.
4.1. High energy intermediates.	4.1.
4.2. Inhibitors of oxidative phosphorylation.	4.5.
4.3. Arsenate and the respiratory chain.	4.7.
Results	4.9.
4.4. Biological and physical properties of aurovertin.	4.9.
4.5. <sup>32</sup> Pi incorporation experiments.	4.11.
4.6. Discussion and conclusions.	4.16.

CHAPTER 5. THE INTERACTION OF DICYCLOHEXYLCARBODI-IMIDE WITH YEAST SMP.

	<u>INTRODUCTION.</u>	5.1.
5.1.	DCCD interactions with mitochondrial membranes.	5.1.
5.2.	Bacterial membrane studies using DCCD.	5.3.
5.3.	Mechanism of carbodi-imide inhibition.	5.5.
	<u>Results</u>	5.6.
5.4.	DCCD inhibition of yeast SMP ATPase activity.	5.6.
5.5.	The interaction of beef heart SMP and ( <sup>14</sup> C) - DCCD.	5.7.
5.6.	The interaction of yeast SMP and ( <sup>14</sup> C) - DCCD.	5.8.
5.7.	Discussion and conclusions.	5.14.

CHAPTER 6. THE PREPARATION AND PROPERTIES OF A SOLUBLE OLIGOMYCIN SENSITIVE ATPase FROM YEAST SMP.

	<u>INTRODUCTION.</u>	6.1.
6.1.	Oligomycin insensitive ATPase preparations.	6.1.
6.2.	Oligomycin sensitive ATPase preparations.	6.2.
6.3.	Other mitochondrial membrane factors.	6.4.
	<u>Results.</u>	6.6.
6.4.	Preparation of an oligomycin sensitive ATPase.	6.6.
6.5.	Properties of the OS ATPase complexes.	6.8.
6.6.	Comparative analysis of the components present in the OS ATPase complexes.	6.11.
6.7.	Discussion and conclusions.	6.14.

CHAPTER 7. THE ROLE OF STEROLS IN THE OLIGOMYCIN SENSITIVITY OF THE MITOCHONDRIAL ATPase.

	<u>INTRODUCTION.</u>	7.1.
7.1.	Sterols and respiratory competency in yeast.	7.1.
7.2.	Yeast mutants and sterol metabolism.	7.2.
	<u>Results</u>	7.4.
7.3.	Ergosterol content of the yeast SMP.	7.4.
7.4.	Saponification of the OS ATPase.	7.5.
7.5.	Selective labelling of yeast mitochondrial lipids.	7.7.
7.6.	Solvent extraction of yeast SMP.	7.7.
7.7.	Discussion and conclusions.	7.10.

CHAPTER 8. DISCUSSION AND CONCLUSIONS.

8.1.
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## SUMMARY

The biochemistry of certain oligomycin resistant mutants of the yeast Saccharomyces cerevisiae D22 has been studied. During the course of this investigation various techniques have been used and as a result of one of the methods used ideas have been proposed on the mode of oligomycin and aurovertin inhibition of mitochondrial energy linked functions.

The interaction of DCCD with yeast SMP membranes has also been studied and a mechanism for the inhibition of yeast SMP ATPase activity is proposed. This mechanism closely resembles the inhibition of red blood cell ATPase activity by carbodi-imides but it is different from the postulated mechanism of DCCD inhibition of beef heart mitochondrial ATPase activity.

Both yeast SMP membranes and a soluble oligomycin sensitive ATPase from these SMP have been used for a comparative study of the parental and oligomycin resistant strains. No gross changes were evident in the whole SMP membrane structure, but there were differences seen in the local lipid environment around the ATPase complex. These differences appeared to be related to differences in the oligomycin sensitivity of the ATPase complex. The importance of lipids within the ATPase complex is further illustrated by a new solvent extraction technique which removes oligomycin sensitivity from the SMP ATPase activity.



# LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ANS	1-anilino-8-naphthalene sulphonate
As <sub>i</sub>	Inorganic arsenate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
DCCD	Dicyclohexylcarbodi-imide
DCU	Dicyclohexyl urea
DNP	Dinitrophenol
DCC	Deoxycholate
EDAC	1-ethyl-3- (3-dimethyl aminopropyl) carbodi-imide
EDTA	Ethylenediamine tetra-acetic acid
F <sub>1</sub>	Soluble ATPase
GEE	Glycine ethyl ester
GLC	Gas Liquid Chromatography
HBHM	Heavy beef heart mitochondria
MW	Molecular Weight
NAD <sup>+</sup>	Nicotinamide adenine dinucleotide
NADH <sub>2</sub> <sup>+</sup>	Nicotinamide adenine dinucleotide (reduced)
Olig.	Oligomycin
OS ATPase	Oligomycin sensitive ATPase
PCA	Perchloric acid
P <sub>i</sub>	Inorganic phosphate
QO <sub>2</sub>	Respiratory quotient
RLM	Rat liver mitochondria
CDS	Sodium dodecyl sulphate
SMP	Sub-Mitochondrial Particle(s)
TEMED	N.N.N'N' Tetramethyl 1.2 diamino ethane

TU	Trypsin urea particles
TTFB	4, 5, 6, 7 tetrachloro-2'-trifluoromethylbenzimidazole
U.V.	Ultra-violet
w/v	Weight by volume
w/w	Weight by weight
X~I	High energy non-phosphorylated intermediate
X~P	High energy phosphorylated intermediate

## CHAPTER I

### INTRODUCTION

The work presented in this thesis is part of a study carried out on the biochemistry of various oligomycin resistant mutants of the yeast Saccharomyces cerevisiae D22.<sup>1</sup> Oligomycin is a macrolide antibiotic,<sup>2</sup> which inhibits oxidative phosphorylation in mitochondria by acting on the membrane-bound ATPase complex. The yeast mutants were isolated by their ability to grow in the presence of oligomycin after the parental strain had been irradiated with ultra-violet light.<sup>1</sup> Both oligomycin - specific mutants and mutants cross resistant to other inhibitors of oxidative phosphorylation such as aurovertin and tri butyl tin chloride have been isolated using this technique.<sup>3</sup> The yeast strains studied so far have been D22 A12, a class 1b mutant, cross resistant to other inhibitors and not temperature sensitive in this resistance, and also strains D22 A16 and D22 A21. These two strains are both specifically resistant to oligomycin,<sup>3</sup> and it has recently been shown that they are allelic cytoplasmic mutants, (P. R. Avner to be published).

Extrachromosomal inheritance of oligomycin resistance has been reported in other yeast strains,<sup>4</sup> but these mutants did not display resistance to oligomycin when the ATPase activity of the isolated mitochondria was tested for oligomycin sensitivity. It was concluded that these yeast strains were resistant by virtue of a change in their permeability to oligomycin or possibly by a detoxification process.

However, with the mutants of Saccharomyces cerevisiae D22 oligomycin resistance has been observed using isolated mitochondria in both the ATP - P<sub>i</sub> exchange and the ATPase reactions.<sup>3</sup>

These observations suggest that some alteration in the mitochondrial membrane itself must have been responsible for the oligomycin resistance in these mutants.

It is hoped that by examining these mutants some indication may be gained as to the role of various membrane fractions in determining mitochondrial oligomycin sensitivity and thus a further insight into the mechanisms of oxidative phosphorylation.

It has been shown that strains D22 A16 and D22 A21 do not differ from the parental D22 WT strain in either their growth rates or final growth yields.<sup>3</sup>

This observation suggests that any membrane change that has occurred in these mutants must be a specific determinant of oligomycin sensitivity, since a widespread membrane alteration would probably change the coupling efficiency of the mutant mitochondria which would be detected in the relative growth rates of the mutant and the WT yeast strains.

Watson and Linnane,<sup>5</sup> have recently examined the morphology of isolated mitochondria derived from other oligomycin resistant strains of Saccharomyces cerevisiae. Using negative-staining electron microscopy they could find no difference in the gross structure of the mitochondrial membranes from the resistant strains. They noted, however, that oligomycin resistance of the mutant strains could be lowered by altering the growth conditions which change both the organization and lipid content of the mitochondrial membranes.<sup>5</sup>

The work presented in this thesis has used a variety of techniques to investigate the mutants of Saccharomyces cerevisiae D22.

An initial examination of the yeast strains at the sub-mitochondrial particle level failed to reveal any significant differences between the mutants and the parental WT strain.

The next approach was suggested by the observations of Cross, Cross and Wang,<sup>6,7</sup> concerning the differential incorporation of  $^{32}\text{Pi}$  into phosphorylating mitochondria which had been inhibited by either aurovertin or oligomycin. These experiments purport to show a method for the study of high energy phosphorylated intermediates synthesized during the generation of ATP. Not only are these experiments interesting in that they possibly demonstrate the existence of such intermediates, but they may also offer an approach to finding the mode of action of oligomycin on the mitochondrial membrane, and as such may help in determining the nature of oligomycin resistance in the yeast mutants.

Another method of examining the oligomycin resistant mutants was the use of dicyclohexylcarbodi-imide,<sup>8</sup> an inhibitor of mitochondrial energy linked functions which apparently has the same inhibitory effects as oligomycin.

However, using beef heart mitochondria, it has been shown that ( $^{14}\text{C}$ ) - DCCD binds covalently to the mitochondrial membranes,<sup>9</sup> unlike oligomycin which is easily washed off the mitochondria.<sup>10</sup> Thus ( $^{14}\text{C}$ ) DCCD was used to investigate the binding and mode of action of DCCD on yeast SMP, with a view to its use as a method of comparing the WT and mutant yeast strains.

A more empirical approach was also tried by preparing soluble oligomycin-sensitive ATPase complexes from the yeast strains and using these as a basis for comparative analyses. By collating biochemical and genetical data with results of the analysis of the composition of the oligomycin sensitive complex it might be possible to determine if subtle changes in the composition of the mutant ATPase complexes were connected to changes in the oligomycin sensitivity.

Swanljung et al<sup>11</sup> have used this technique to investigate the oligomycin resistant mutants Saccharomyces cerevisiae D22 A16 and D22 A21. They report a lower concentration of ergosterol in both the whole yeast cells and in a soluble oligomycin sensitive ATPase complex derived from the mutant strains compared to D22 WT. These findings are discussed in Chapters 6 and 7, where measurements of the ergosterol content of both the yeast SMP and an oligomycin sensitive ATPase complex are presented.

Also given in Chapter 7 are results of a solvent extraction technique which removes the oligomycin sensitivity of the membrane bound ATPase. Further development of this technique may well give more information on the exact role of sterols in determining the oligomycin sensitivity of the ATPase complex.

Since the results presented in this thesis cover various distinct aspects of oxidative phosphorylation a separate introduction to each topic is presented at the beginning of each chapter.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1. Materials

All the chemicals used were of the highest grade possible. Normal laboratory reagents were obtained from BDH. Chemicals Ltd. ( $^{14}\text{C}$ ) DCCD was synthesized at Sittingbourne by Mr. P. Haarthorn. ( $^{14}\text{C}$ ) GEE was obtained from N.E.N. Industries Ltd.  $^{32}\text{Pi}$  was obtained from the Radiochemical Centre, Amersham. Oligomycin was obtained as a mixture of oligomycins A and B from Sigma Chemicals.

#### 2.2. Growth of Yeast

Yeast cultures were grown in a CeCa fermentor (Gallenkamp Ltd.) in 4 litre batch cultures. The medium used was 2% (w/v) glucose, 2% (w/v) bacteriological peptone and 1% (w/v) yeast extract. 0.05% (v/v) tributyl citrate was added to the medium prior to autoclaving as an antifoam agent. The cultures were grown at  $30^{\circ}\text{C}$  for 40 hours with the paddle stirrer turning at 600 rpm. Since the vortex-type aeration was found to be ineffective the apparatus was modified to include a sparger through which air was pumped at 500 ml/min/litre of culture.

The constant pH facility on the apparatus was not used.

50 ml. of a 24 hour yeast culture was used to inoculate the fermentor.

#### 2.3. Preparation of SMP from the cultured cells

All steps in this preparation were performed at  $0^{\circ}\text{C}$ .

The cells were harvested by centrifuging the culture fluid at 2,000 g x 10 mins. The cells were washed once with distilled water and recentrifuged at 2,000 g x 10 mins. The washed yeast pellet was weighed and then the yeast cells were resuspended in

0.25 M sucrose, 0.1 M. Tris Cl pH 7.5 (buffered sucrose) at 1:1 (w/v) ratio of wet weight cells to buffered sucrose.

30 ml. of this cell suspension was placed in a 90 ml. capacity Braun glass bottle with 30 gm. of 0.18 - 0.22 mm. diameter Balotini glass beads. The bottle was then shaken for 25 seconds at speed II in a Braun shaker using liquid CO<sub>2</sub> as a cooling agent.

The supernatant was decanted from the glass beads and these were then washed twice with buffered sucrose. These washings and the supernatant were combined and then centrifuged for 2,000 g x 20 mins. The pellet was discarded and then the supernatant was centrifuged at 20,000 g x 20 mins.

The crude mitochondrial pellet was resuspended in buffered sucrose and then centrifuged at 4,000 g x 10 mins. The pellet was discarded and the supernatant centrifuged at 20,000 g x 20 mins.

The mitochondrial pellet was resuspended in buffered sucrose at a protein concentration of 25 mg/ml and then sonicated for 60 seconds in a MSE sonicator adjusted for maximum power.

The sonicated suspension was centrifuged at 25,000 g x 20 mins. and the pellet discarded.

The supernatant was then centrifuged at 120,000 g x 30 mins. to collect the yeast SMP. The supernatant was discarded and any lipid adhering to the sides of the centrifuge tube was removed.

After washing the surface of the SMP pellet twice the SMP were resuspended in buffered sucrose at a protein concentration of approximately 40 mg/ml.

#### 2.4. Preparation of Rat Liver Mitochondria

All operations were performed at 0°C.

An adult rat was sacrificed and then the liver was rapidly removed and placed in 100 ml. of 0.25 M. sucrose, pre-cooled to 0°C.



The liver was cut into small segments and these were then washed three times with 0.25 M sucrose.

The liver pieces were homogenized in 25 ml. of 0.25 M sucrose by a power-driven Potter homogenizer. The homogenate was centrifuged at 1,500 g x 10 mins. The top  $\frac{2}{3}$  rds. of the supernatant was carefully removed and centrifuged at 10,000 g x 10 mins. The supernatant was discarded and the pellet surface washed with 0.25 M sucrose. The mitochondrial pellet was then resuspended in a small volume of 0.25 M sucrose, avoiding disturbing any red blood cells at the base of the mitochondrial pellet. The final protein concentration was approximately 50 mg/ml.

#### 2.5. Preparation of Beef Heart Mitochondria

Heavy beef heart mitochondria (HBMH) were prepared by 'Nagarse' enzyme digestion technique described by Smith.<sup>15</sup>

#### 2.6. Lipid extraction by chloroform-methanol

Lipid extractions were performed by adding 20 volumes of a chloroform-methanol (2:1, v/v), mixture to the sample. The suspension was filtered through a glass sinter and then the filtrate was washed with distilled water,  $\frac{1}{5}$ th. of the volume of the chloroform-methanol solution being used. The organic phase was removed after the two phases had been separated by centrifuging at 1,000 g for 1 hour at  $-10^{\circ}\text{C}$ .

The solvents from the organic phase were removed under reduced pressure and then the samples were redissolved in a minimum volume of chloroform-methanol (2:1, v/v).

#### 2.7. Thin Layer Chromatography

All the work was performed using pre-coated TLC plates supplied

by E. Merck Ltd. They were activated at  $120^{\circ}\text{C}$  for 1 hour immediately prior to their use.

The system used for separating the various aurovertin fractions was on silica gel plates, 2.0 mm. thick for preparative work and 0.25 mm. thick for analytical runs. Chloroform-acetone (7:3, v/v), was used as a solvent system. The aurovertin spots were developed by spraying the plates with 50%  $\text{H}_2\text{SO}_4$ .

The system used for separating DCCD and DCU was on 0.25 mm. alumina plates using benzene-ethyl acetate, (9:1, v/v), as a solvent system.

When radioactive material was being used the plates were scanned with a Berthold series 600 scanner.

Cold DCCD and DCU were detected by exposing the plates to iodine vapour or by examination under U.V. light.

Lipid analyses were performed on 0.25 mm. silica gel plates. The phospholipid fractions were separated using a chloroform-methanol- aqueous, (32:5, w/v), ammonia, (17:7:1, v/v), solvent system.<sup>16</sup> Molybdenum blue reagent<sup>17</sup> was used to detect the separated phospholipids. The neutral lipid fraction was separated by running the sample on 0.25 mm. silica gel plates, using a petroleum ether ( $60-80^{\circ}\text{C}$ ) - diethyl ether - acetic acid, (90:10:1), solvent system.<sup>18</sup> The fractions were detected by spraying the plates with 50%  $\text{H}_2\text{SO}_4$  and then heating them on a hot plate for 1 - 2 minutes.

## 2.8. Radioactivity Measurements

A Triton : Toluene : BPD scintillator was used. 4.66 gm. of BPD were added to a litre of a 2:1 (v/v) mixture of Toluene - Triton X-100. Measurements of radioactivity were normally

made for 10 minute counting periods in either a Packard Tri-Carb liquid scintillation spectrometer or an Intertechnique scintillation counter. A channel ratio technique was used to correct  $^{14}\text{C}$  cpm to dpm.

## 2.9. Polyacrylamide Gel Electrophoresis

Two electrophoresis systems were used in this thesis, the first was described by Weber and Osborn,<sup>19</sup> except that only half the amount of bisacrylamide was used in the polyacrylamide gels. This was found to give better separation of the proteins in the OS ATPase complex. However, this system proved unsuitable for full solubilization of the yeast SMP membranes and another SDS polyacrylamide gel system was developed.

The details of the second system are given below;

Stock polyacrylamide solution	(	10% (w/v) acrylamide
	(	0.3% (w/v) bisacrylamide
	(	0.1M glycine - NaOH pH 10.7
	(	0.1% (w/v) SDS
Reservoir solution	(	50 mM glycine - NaOH pH 10.7
	(	1% SDS

To polymerize the polyacrylamide 0.5 ml. of 0.15% (w/v) ammonium persulphate and 40  $\mu\text{l}$  of TEMED were added to 20 ml. of the stock polyacrylamide gel solution.

The samples to be electrophoresed in this system were depolymerized by taking 0.25 ml. of an approximately 3 mg. protein/ml. sample solution and adding to it 0.25 ml. of 0.1M phosphate buffer at pH 11.5. To this 0.25 ml. of the second depolymerizing solution was added.

Second depolymerizing	(	3% (w/v) SDS
solution	(	4% (w/v) mercaptoethanol
	(	5 mM EDTA
	(	5 mM dithiothreitol

50  $\mu$ l of glycerol and a drop of 0.5% (w/v), bromophenol blue were added to the 0.75 ml. of depolymerized sample.

100  $\mu$ l of this final solution was then layered onto each gel and the system was then electrophoresed at 5 mA/gel until the tracker dye was within 0.5 cm. of the bottom of the polyacrylamide gels.

With both this gel system and the Weber and Osborn system the fixing and staining procedure was the same. This consisted of an overnight fix in 10% (v/v) ethanol, 10% (v/v) acetic acid. The gels were removed and then placed in the staining fluid for 1½ hours.

Staining	(	0.25% (w/v) Coomassie Blue
fluid	(	45% (v/v) ethanol
	(	10% (v/v) acetic acid.

The gels were destained, over a period of 2 to 3 days, using the fixing solution.

## 2.10. ATPase Measurements

Two methods were used for measurement of ATPase activity and the oligomycin sensitivity of this activity. Both methods gave similar results for the ATPase activity and oligomycin sensitivity of this activity, the assays being performed at pH 9.5 and 30°C.

The method normally used, since it was quicker and required less sample material, was that described by Robertson *et al*<sup>20</sup> in which the hydrolysis of ATP is linked to the oxidation of  $\text{NADH}_2^+$ . The disappearance of  $\text{NADH}_2^+$  was followed at 366 nm, KCN is included in the incubation medium to prevent oxidation of the  $\text{NADH}_2^+$  by

respiratory chain activity.

The second assay system is based on an assay of the phosphate released during ATP hydrolysis.<sup>21</sup> This method was not suitable for the soluble oligomycin sensitive ATPase preparations since it required more material than the  $\text{NADH}_2^+$  - linked assay and also took longer to perform. Since some assays were being performed using an unstable OS ATPase preparation the quicker method was an obvious choice.

However, using yeast SMP, both assays gave similar results so the  $\text{NADH}_2^+$  linked method was used for all the results presented in this thesis. No preincubation was used in determining the oligomycin sensitivity of the ATPase activity, the ATPase sample and appropriate amount of oligomycin were added together in the cuvette, prior to the ATPase assay.

### 2.11. Oxygen Electrode Measurements

A Clarke oxygen electrode maintained at  $25^\circ\text{C}$  was used for measuring  $\text{ADP}/0$  and respiratory control ratios. The basic electrode buffer used for both rat liver and beef heart mitochondria was:

Electrode	(	15 mM KCL
buffer	(	50 mM Tris $\text{SO}_4$ pH 7.5
	(	1 mM EDTA
	(	2.5 mM $\text{MgCl}_2$

The electrode vessel had a 3.5 ml. volume, additions to this being 200  $\mu\text{l}$  of 1.0 M substrate solutions, these being either succinate or a mixture of malate and glutamate.

25  $\mu\text{l}$  of 40 mM ADP was added in respiratory control ratio experiments. For the phosphate/arsenate experiments 25  $\mu\text{l}$  of 1.5 M pH 7.5 phosphate buffer or 50  $\mu\text{l}$  of 1.4 M arsenate solution were added to the electrode vessel.

## 2.12. Pyridine Nucleotide Transhydrogenase Measurement

Measured as described by Beechey et al.<sup>8</sup>

## 2.13. Protein Estimation

For protein concentrations greater than approximately 5 mg/ml. the biuret method of Gornall et al.<sup>22</sup> was used, after the sample had been solubilized with 1.5% sodium deoxycholate.

For protein solutions less than 5 mg/ml. the Folin - Ciocalteu method was used.<sup>23</sup>

## 2.14. Phosphate Assays

The total phosphate content of a sample was determined by an initial treatment with excess conc.  $\text{H}_2\text{SO}_4$  at  $160^\circ\text{C}$ , for 24 hours.

The carbonized samples were oxidized with Anlar  $\text{H}_2\text{O}_2$ , at  $160^\circ\text{C}$ , until they were colourless. Heating was continued a further 6 hours and then the phosphate content was determined by the method of Chen et al.<sup>24</sup>

For inorganic phosphate determinations in mitochondria or P.C.A. mitochondrial precipitates the initial charring and oxidization steps were omitted.

## 2.15. Extraction of Aurovertin

The aurovertin extracted at Sittingbourne from the Calcasporium Sp was designated S.aurovertin to distinguish it from the original aurovertin supplied by the Pitman - Moore division of the Dow Chemical Co.

The extraction procedure used was the same as that described by Baldwin et al.<sup>25</sup> except that the final purification was by preparative TLC, as described previously, and not counter current distribution.

### 2.16. Ether Extraction of Yeast SMP

Yeast SMP, at 30 mg. protein/ml. in buffered sucrose, were shaken with 1 ml. of diethyl ether/mg. SMP protein at room temperature. The two layers were allowed to separate and then the ether layer removed. The extraction was repeated twice. After the final extraction traces of ether remaining in the extracted SMP were removed by using a Buchi rotatory evaporator for 1-2 minutes. The extracted yeast SMP were then resuspended to their original volume with 0.25 M sucrose, 0.1 M. Tris. Cl pH 7.5. These extracted SMP were then stored at  $-27^{\circ}\text{C}$ .

### 2.17. Saponification and GLC Techniques

0.2 ml. samples of the yeast SMP or OS ATPase preparations were refluxed for 1 hour at  $100^{\circ}\text{C}$  with 10 ml. of 10% (w/v) KOH, 10% (v/v) ethanol. After cooling this solution was extracted three times with 20 ml. of petroleum ether ( $40-60^{\circ}\text{C}$ ). These ether extracts were then combined and washed once with 20 ml. distilled water. The organic phase was removed and dried overnight over 1 gm. of anhydrous sodium sulphate.

After removing the sodium sulphate the ether extract was evaporated to dryness under a  $\text{N}_2$  stream. The samples were redissolved in 0.2 ml. of diethyl ether and stored in the dark at  $-27^{\circ}\text{C}$ .

The GLC was performed on a Varian Aerograph Series 1800 machine using a 4% JXR liquid phase on a Gas Chrom Q support in a  $3' \times 1/8''$  column.  $\text{N}_2$  was the gas phase with a flow rate of 50 ml/min. A programmed technique was found to give the best peak separation, the programme starting at  $200^{\circ}\text{C}$ , holding for five minutes and then rising to  $250^{\circ}\text{C}$  at  $5^{\circ}\text{C}/\text{min}$ .

## CHAPTER 3

### A COMPARATIVE STUDY OF SMP FROM D22 WT AND VARIOUS OLIGOMYCIN RESISTANT STRAINS

#### 3.1. Introduction

This work was performed to see if oligomycin resistance in the mutant S.cerevisiae strains had entailed an obvious alteration in mitochondrial membrane structure. An earlier study,<sup>12</sup> had indicated that there may be a difference in the acid-soluble membrane proteins of mitochondria from the oligomycin-resistant strains. However, this technique was limited in its application since not all the membrane was solubilized, and therefore the lack of certain acid soluble proteins seen using polyacrylamide gel electrophoresis could have been due to either a genuine protein deletion or to another alteration which affected the relative acid-solubility of these proteins.

Recently Swanljung et al<sup>11</sup> have published a report claiming that there is a lower concentration of ergosterol in both the whole yeast cells and in a soluble oligomycin sensitive ATPase complex derived from the oligomycin resistant mutants D22 A16 and D22 A21. However, it was found that growth of these mutants in the presence of ergosterol did not affect the oligomycin sensitivity of the SMP ATPase (P. Swanljung, personal communication).

Low temperature difference spectra of the yeast cytochromes have failed to reveal any differences between the D22 WT and mutant strains, (M. Skipton, unpublished observations).



## Results

### 3.2. Yeast Culture and Growth

A typical growth curve of S.cerevisiae D22 WT is shown in Fig. 3.1. The optical density of the culture and also the  $QO_2$  values of the cells are plotted against time of growth. It can be seen there is a typical biphasic growth pattern, the yeast developing maximum respiratory ability during the second, oxidative phase of growth.<sup>13</sup> The oligomycin resistant strains D22 A16 and D22 A21 show no difference from the parental D22 WT in either growth rates or final growth yields, (P. R. Avner to be published). To obtain yeast cells with good respiratory and mitochondrial functions all the cultures were grown for 36-40 hours, when the cellular  $QO_2$  value reaches a maximum and the cells are fully glucose derepressed. With the growth medium described in Chapter 2 yields of 30-35 gm. wet weight yeast cells, per litre of culture, were obtained. The yield of SMP protein was 3-4 mg/gm. wet weight yeast cells. No significant variation was seen in the yield of SMP with any of the strains used.

### 3.3. Oligomycin sensitivity of the SMP ATPase

The specific activity of the yeast SMP ATPase varied from 0.5 to 1.7  $\mu$ moles/min/mg. but normally the SMP had ATPase activities of approximately 1.0  $\mu$ mole/min/mg. There was no correlation between ATPase activity and the oligomycin sensitivity of the ATPase activity.

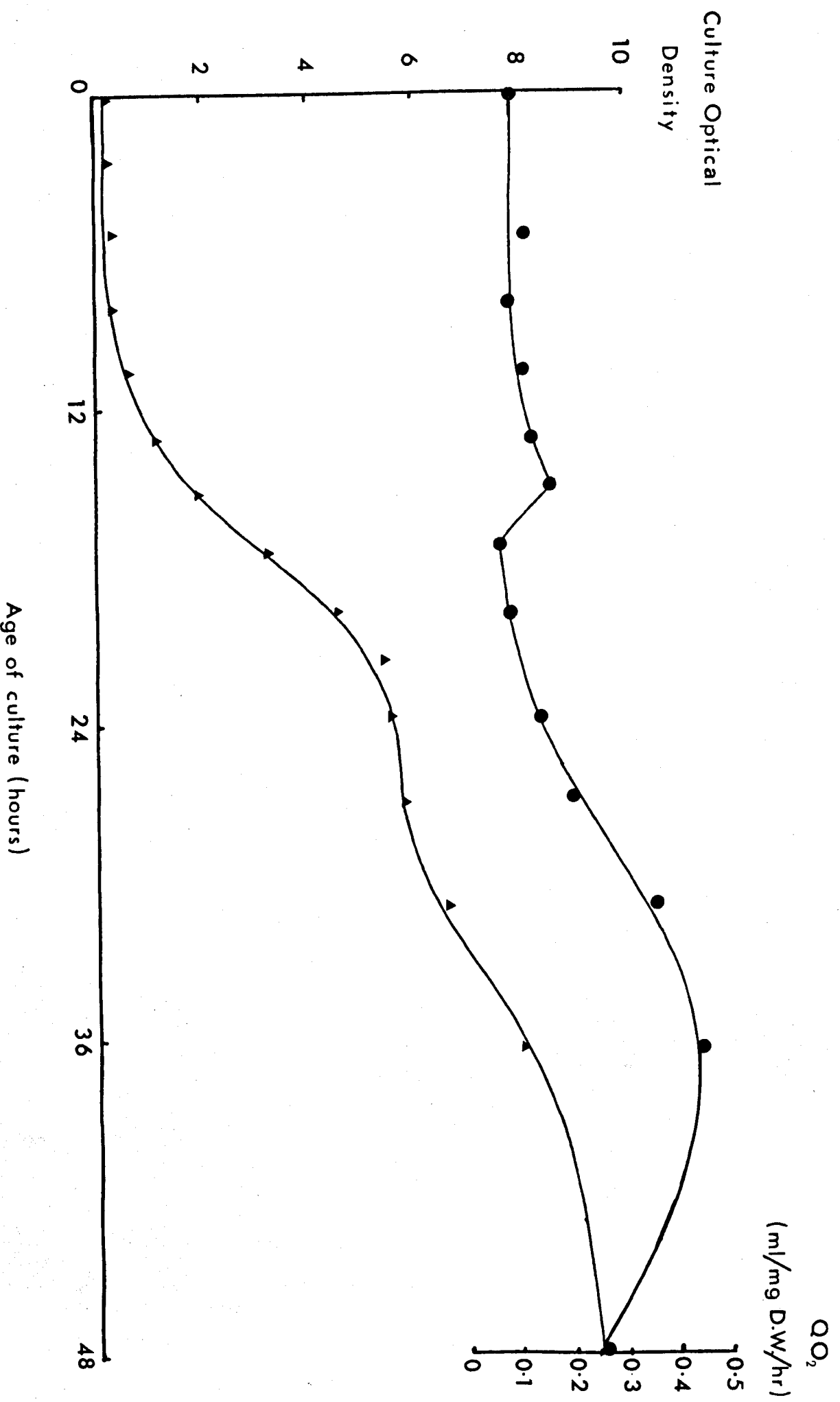
Oligomycin inhibition curves of the SMP ATPase activity are shown in Fig. 3.2. This shows that strains D22 and A16 and

FIGURE 3.1.

GROWTH CURVE OF S.CEREVISIAE D22 WT

The growth of S.cerevisiae D22 WT at 30°C is shown by the culture optical density, at 607 nm, the conditions of growth are as described in Chapter 2, section 2.2. Also illustrated is the cellular respiratory quotient,  $QO_2$ . The  $QO_2$  of the washed yeast cells in 50 mM phosphate buffer, pH 7.0 plus 2% glucose is expressed in ml.  $O_2$  consumed per mg. dry weight yeast cells per hour.

- :  $QO_2$  cells (ml. $O_2$ /mg. dry weight/hour)
- ▲ : Optical density of culture at 605 nm.



D22 A21 have very similar sensitivities to oligomycin. The range of 50% inhibition concentrations for D22 A16 was 20.0-22.0  $\mu\text{g}$  oligomycin/mg. protein and for D22 A21 the 50% inhibition concentrations were 21.0-25.0  $\mu\text{g}$  oligomycin/mg. protein.

D22 A12 had a higher sensitivity to oligomycin, the 50% inhibition concentrations being 7.0-8.0  $\mu\text{g}$  oligomycin/mg. protein. These mutants were all more resistant to oligomycin than the D22 WT which was usually found to be inhibited by 1.5  $\mu\text{g}$  oligomycin/mg. protein. However, the D22 WT 50% inhibition value was more variable than in the mutant strains. Although normally 50% inhibited by 1.5  $\mu\text{g}$  oligomycin/mg. protein the range of values found was 0.5-4.0  $\mu\text{g}$  oligomycin/mg. protein. This variation in the sensitivity of the D22 WT SMP ATPase to oligomycin has been found by other workers who have calculated 50% inhibition values ranging from 1.0-5.0  $\mu\text{g}$  oligomycin/mg. protein, (P. Swanljung, personal communication). This large variation in oligomycin sensitivity was not seen with the mutants D22 A12, D22 A16 or D22 A21.

Since the yeast strains were grown under identical cultural conditions there is no apparent reason for the observed variation of the D22 WT sensitivity to oligomycin, unless this variation is linked to the degree of oligomycin sensitivity shown by the SMP ATPase.

FIGURE 3.2.

OLIGOMYCIN INHIBITION CURVES OF SMP ATPase ACTIVITY

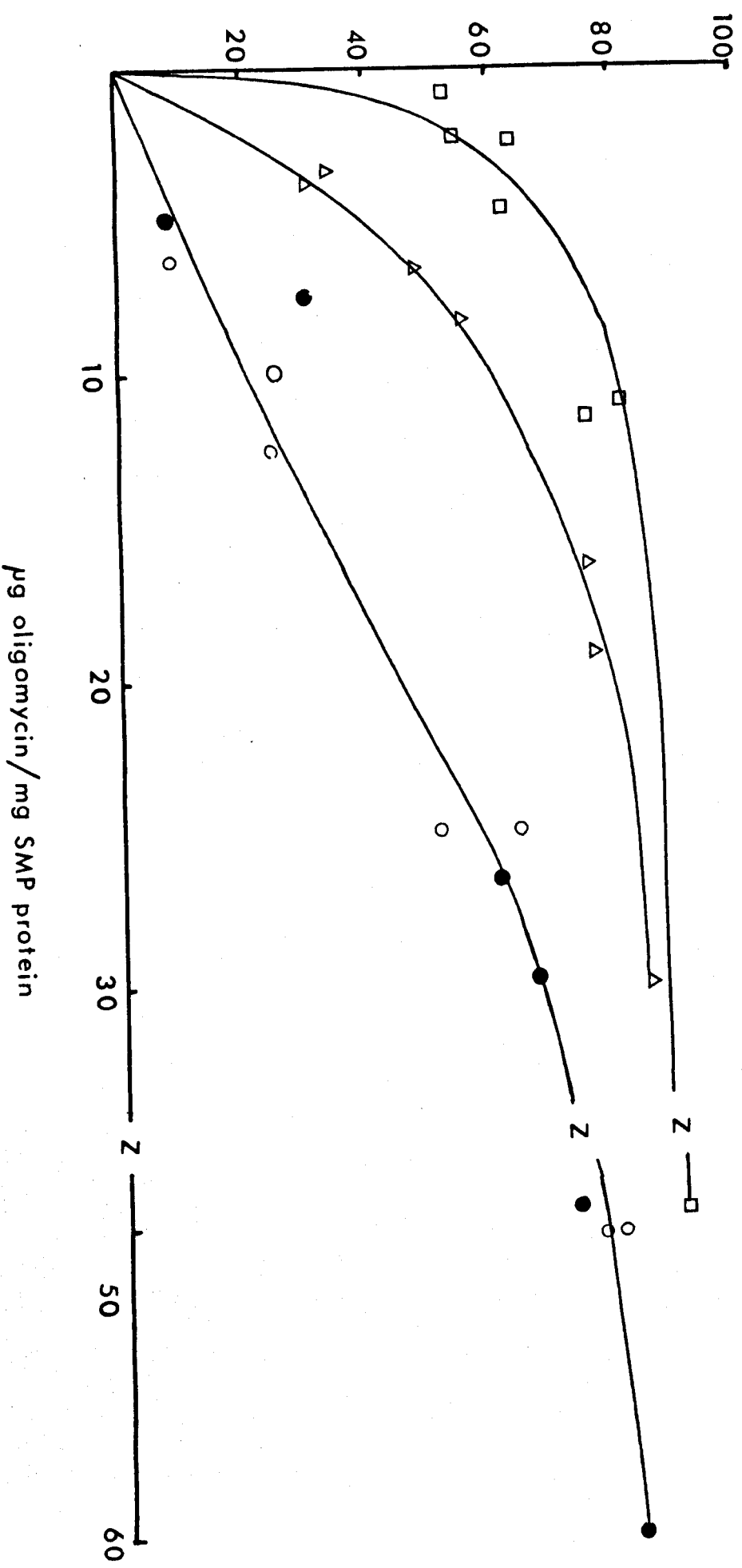
The ATPase activity was measured at pH 9.5 and 30°C by the NADH oxidation method described in Chapter 2, section 2.10. No preincubation period was used to measure the oligomycin inhibition of the ATPase activity.

The curve for each yeast strain is plotted through the points obtained from two separate preparations of yeast SMP.

The ATPase specific activities of the SMP were all within the range 0.57 to 1.7  $\mu\text{moles/min/mg}$ .

□	:	D22 WT,	ATPase Specific Activity	:	0.6 and 1.0 $\mu\text{moles/min/mg}$ .
Δ	:	D22 A12,	"	:	0.9 and 1.6 $\mu\text{moles/min/mg}$ .
●	:	D22 A16,	"	:	0.9 and 1.1 $\mu\text{moles/min/mg}$ .
○	:	D22 A21,	"	:	0.6 and 1.4 $\mu\text{moles/min/mg}$ .

% Inhibition of  
ATPase



The SMP from all the strains were stable, as judged by ATPase activity and the oligomycin sensitivity of this activity, when stored at  $-27^{\circ}\text{C}$ .

It should be noted that none of the three mutants is resistant to oligomycin in an absolute sense, i.e., the SMP ATPase activity can be over 90% inhibited by addition of sufficient oligomycin.

### 3.4. Polyacrylamide Gel Electrophoresis of the SMP

As mentioned before an earlier study,<sup>12</sup> had used an acid extraction technique to examine the mitochondrial membrane proteins but since the membranes were not completely solubilized it is difficult to draw definite conclusions on the disappearance of certain protein bands using a polyacrylamide gel electrophoresis technique.

Techniques published for solubilizing beef heart mitochondria<sup>9</sup> were found to be inadequate for completely solubilizing the yeast SMP, since a layer of protein remained on the top of the gels after electrophoresis.

Homogenizing the yeast SMP in 8 M urea at pH 10.5 also failed to give satisfactory depolymerization of the membranes. However, using the SDS/Glycine - NaOH electrophoresis system described in Chapter 2 complete membrane solubilization was achieved and the results of this electrophoresis technique are shown in Figure 3.3. This shows gels of SMP from D22 WT, D22 A12, D22 A16 and D22 A21. It can be seen that although a very complex pattern of protein bands is given there is no consistent or obvious alteration in the mutant strains compared to D22 WT, also no protein remains on the gel surface, indicating that the method is suitable for comparison of the protein composition of the SMP membrane. Gel scanning has

FIGURE 3.3.

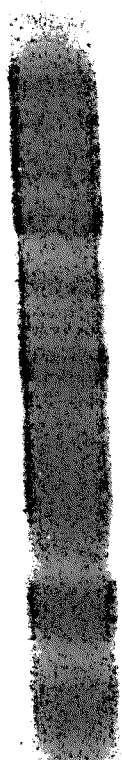
POLYACRYLAMIDE GELS OF YEAST SMP

The gels shown are of yeast SMP in the SDS/Glycine - NaOH system described in Chapter 2, section 2.9. The gels shown, left to right, SMP from D22 WT, D22 A12, D22 A16 and D22 A21 yeast strains.

100-150  $\mu$ g protein was applied to each gel.



⊖



- front

⊕

WT

A12

A16

A21

confirmed the identical nature of the protein components in the SMP from the various yeast strains. Minor protein bands do not show up well in Figure 3.3, but examination of the gels disclosed no obvious differences in the mutant strains compared to D22 WT.

### 3.5. Lipid Analysis of the SMP

#### (a) Phospholipids.

Using the chloroform-methanol extraction technique described in Chapter 2 the phospholipid contents of the SMP were compared analysing the extract on the chloroform-methanol-ammonia TLC system described earlier. Analysis of the TLC plate showed that there were phosphate positive compounds running with the same Rf values as phosphatidyl inositol, phosphatidyl choline and phosphatidyl ethanolamine present in the extracts from D22 WT, D22 A16 and D22 A21 SMP.

Analysis of the total phosphate content of these SMP showed there was no significant difference between the strains, there being 11.5, 13.6 and 14.7 nmoles of phosphate/mg. SMP protein in D22 WT, D22 A16 and D22 A21 SMP. Previous experiments had also failed to find a significant difference in the total phosphate content of mitochondria from the various strains, (P. R. Avner, personal communication).

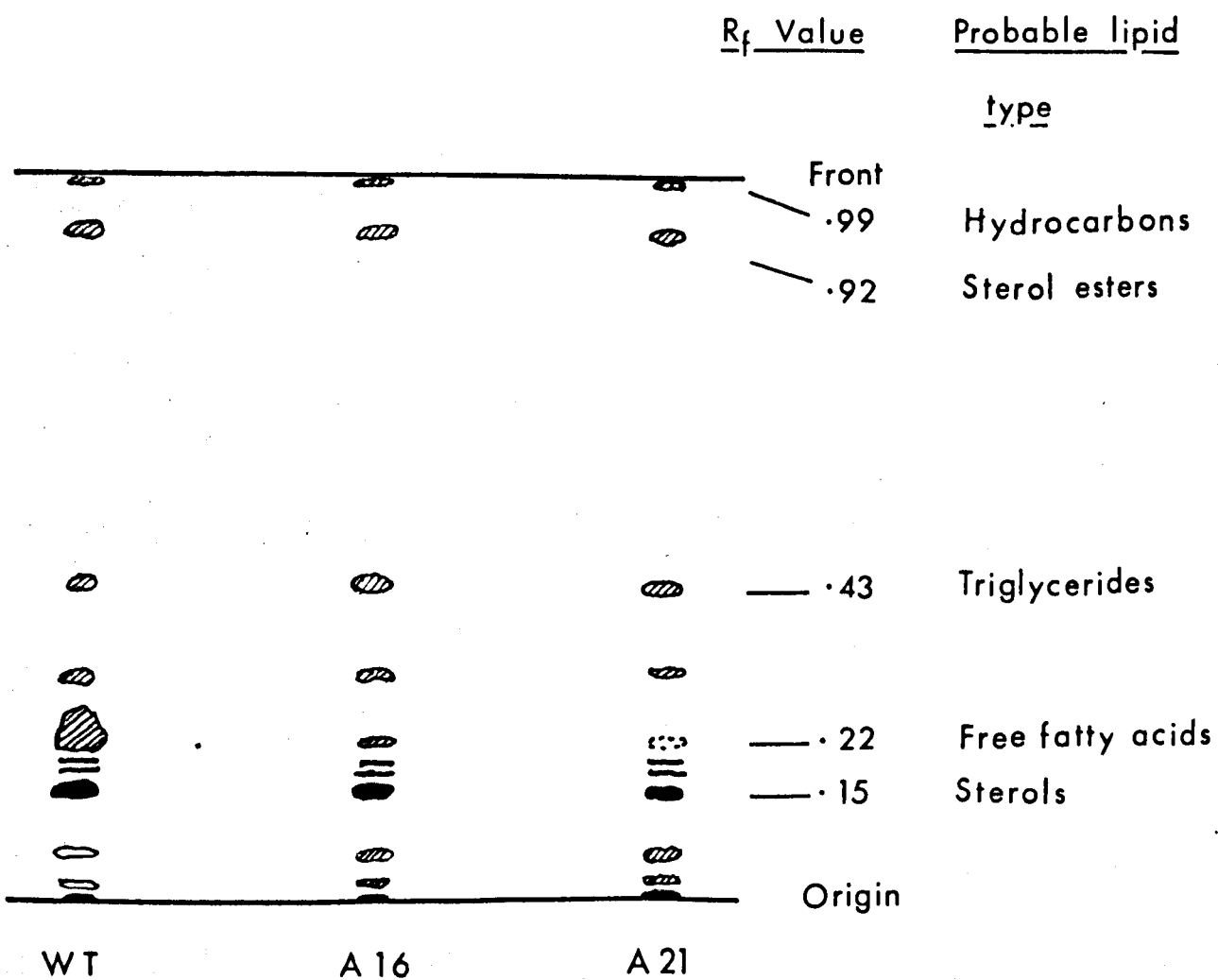
#### (b) Neutral Lipids

Using the same chloroform-methanol extracts that had been used for phospholipid analysis the neutral lipid fraction was separated by TLC using the system described in Chapter 2. A diagram of the TLC plate is shown in Figure 3.4. As can be seen there are many components present in this fraction, compounds running with the same Rf values as esterified ergosterol (0.92), triglycerides (0.443), free

FIGURE 3.4.

THIN LAYER CHROMATOGRAPHY OF THE NEUTRAL  
LIPID FRACTION FROM YEAST SMP.

This shows a TLC analysis of the chloroform-methanol extracts of the yeast SMP using a pet. ether - diethyl ether - acetic acid system as described in Chapter 2, sections 2.6 and 2.7. The samples are left to right, extracts from D22 WT, D22 A16 and D22 A21 SMP. The spots were visualized by charring with a 50%  $\text{H}_2\text{SO}_4$  spray.



fatty acids (0.22) and free ergosterol (0.150), are present as well as other minor components. There does appear to be some difference between the D22 WT chloroform-methanol extract and that from D22 A16 and D22 A21 since compounds running with the same mobility as free fatty acids are much less noticable in the extracts from the mutant SMP.

On the TLC plate there did not appear to be a significant difference between the ergosterol, (compound Rf 0.15), content of the SMP from D22 WT, D22 A16 and D22 A21.

Quantative estimations of the ergosterol content of the yeast SMP were performed using a saponification/GLC technique and these results are presented in Chapter 7 where they are discussed in relation to the report of Swanljung et al<sup>11</sup> concerning the ergosterol content of the mutant yeast.

It was found that the ergosterol content of the SMP did not vary amongst the various strains examined.

### 3.6. Fluorescence Studies with Yeast SMP

In an attempt to detect possible changes in the SMP membrane conformation caused by oligomycin resistance various studies were performed using ANS fluorescence techniques. Although beef heart mitochondria were found to give energy-linked fluorescence changes no such response was obtained using SMP from strains of S.cerevisiae D22. This work was done in conjunction with M. Skipton and full details are given in <sup>14</sup>.

### 3.7. Discussion and Conclusions

The previous observations<sup>3</sup> concerning the resistance of the ATPase activity to oligomycin on SMP isolated from oligomycin

resistant strains of S.cerevisiae D22 have been confirmed.

Experiments have been performed to determine the composition of SMP derived from both the parental D22 WT strain and the oligomycin resistant strains D22 A16 and D22 A21. Using SDS polyacrylamide gel electrophoresis techniques no significant differences could be detected in the various membrane proteins from these three strains, or in another oligomycin resistant strain D22 A12.

Similarly no qualitative differences could be found in the phospholipid components from the D22 WT, D22 A16 or D22 A21 SMP. There was also no significant difference in the total phosphate content of the SMP membranes from these strains.

No difference has been detected in the other lipid components from these strains, except that the free fatty acid content of D22 A16 and D22 A21 appeared to much less than that of D22 WT.

The oligomycin sensitivity of the D22 WT SMP ATPase activity varied considerably more than that from the strains D22 A12, D22 A16 and D22 A21. However, the most "resistant" of the D22 WT SMP ATPase preparations was still more sensitive to oligomycin than any of the mutant SMP preparations. There is no obvious explanation for the variation seen only with the D22 WT preparations, since all the yeast strains were cultured under identical conditions.

The observation that the ATPase of SMP from D22 A16 and D22 A21 had very similar oligomycin sensitivities is significant since these two mutants have recently been shown to be allelic, (P. R. Avner, to be published).

However, the alteration that is responsible for the changes in the oligomycin sensitivity of the mutant SMP ATPase is probably

too subtle to detect by analysing the whole SMP, other membrane components not concerned with the ATPase complex masking any fine alteration in membrane structure.

Therefore, for further comparative analyses of the oligomycin resistant mutants and the D22 WT strain to be successful a more purified membrane complex containing the site of alteration in the SMP membrane must be prepared. The significance of the lack of free fatty acids in the mutant SMP is uncertain, but if a purified oligomycin sensitive ATPase complex also lacked free fatty acids when prepared from the mutant SMP, then this observation may well be linked to the <sup>mode</sup> of oligomycin resistance in the mutant yeast strains.

## CHAPTER 4

### A STUDY OF THE EFFECTS OF AUROVERTIN AND OLIGOMYCIN ON $^{32}\text{P}$ i INCORPORATION INTO MITOCHONDRIA

#### Introduction

##### 4.1. High Energy Intermediates

Cross, Cross and Wang<sup>6,7</sup> have used the inhibitors aurovertin and oligomycin to obtain a differential incorporation of  $^{32}\text{P}$ i into rat liver mitochondria. The aurovertin-inhibited mitochondria incorporated more  $^{32}\text{P}$ i than those mitochondria which had been inhibited with oligomycin. They produce evidence to support the idea that these inhibitors are acting immediately before and after the synthesis of a high energy phosphorylated intermediate, or 'X~P'. The evidence will be discussed later in this introduction.

It was intended to use this technique to compare the capacity for phosphorylation of the D22 WT and mutant mitochondria in an attempt to gain an insight into any alterations in the energy metabolism of the yeast that may have occurred as a consequence of oligomycin resistance.

The experiments themselves may have a significant effect on the elucidation of oxidative phosphorylation.

The concept of high energy bond formation in mitochondria, prior to the synthesis of ATP, was first proposed by Slater in 1953 in his original chemical hypothesis,<sup>26</sup> which postulated high energy forms of respiratory chain carriers. Chance *et al*<sup>27</sup> first provided evidence for the existence of high energy intermediates when they showed that, in the absence of ADP and Pi, succinate added to mitochondria will cause  $\text{NAD}^+$  to be rapidly reduced, i.e., the



respiratory chain was driven backwards, an endergonic process.

This 'respiratory chain reversal' driven by succinate was oligomycin insensitive.<sup>28,29</sup> This reversal may also be accomplished using ATP as an energy source, instead of succinate oxidation.<sup>129</sup> However, it was found that the ATP-driven reversal is sensitive to oligomycin.<sup>30</sup> It appeared therefore that oligomycin was inhibiting coupled respiration in mitochondria by interfering with the conversion of some form of high energy intermediate to ATP. The accumulation of this high energy store in the absence of ATP synthesis has been demonstrated by other methods than the reversal reaction. Tager and Slater<sup>31</sup> have shown that in oligomycin inhibited mitochondria respiration can be restored by the addition of  $\alpha$  ketoglutarate and  $\text{NH}_4^+$ , which are then reduced by  $\text{NADH}^+$  to generate glutamate. This reaction requires some source of energy which is presumably provided in the inhibited mitochondria by the accumulated high energy intermediates.

Studies on the selective accumulation of ions by cells have shown that this is unaffected by oligomycin, but is inhibited by dinitrophenol, which has been considered to discharge the high energy intermediates needed to drive the ion uptake.<sup>32-34</sup> Chance et al<sup>35</sup> have used a similar technique involving  $\text{Ca}^{++}$  uptake to calculate that the high energy store in state 4 mitochondria is equivalent to 0.3 nmoles ATP per mg. mitochondrial protein.

It has also been shown that the pyridine nucleotide trans-hydrogenase reaction is maintained by high energy intermediates rather than ATP.<sup>36</sup>

All these findings support the existence of high energy intermediates which can accumulate in the absence of ATP synthesis.

However, the form in which these intermediate(s) exist has yet to be demonstrated.

There are three principal theories as to the form of a hypothetical high energy non-phosphorylated intermediate.

- (1) A chemical interaction between respiratory chain carriers and various functional groups on the membrane.
- (2) The generation of an electrical potential across the mitochondrial membrane by the transport of  $H^+$ .
- (3) An energy-linked change in the mitochondrial membrane conformation.

The latter, conformational, model has been put forward strongly by Green et al<sup>38</sup>, supported by the observations of Hackenbrock on condensed and open forms of mitochondrial structure.<sup>39</sup>

Green et al<sup>40</sup> have recently described an 'Electromechanochemical' model incorporating all three forms of basic theory on the nature of the high energy non-phosphorylated intermediate. They state in this theory that the mitochondrion is the smallest unit capable of ATP generation coupled to substrate oxidation, since ATP synthesis is dependant on the environment of the internal mitochondrial matrix.

The necessity of a membrane for ATP generation distinguishes the theories of Green and that of the membrane potential from the purely chemical theory. Obviously the isolation of a non-membranous particulate ATP-generating system would weigh heavily in favour of a purely chemical hypothesis, but to date this has not been achieved.

All three theories, however, involve the generation of a high energy phosphorylated intermediate,  $X \sim P$ , from the high energy non-phosphorylated intermediate,  $X \sim I$ .  $X \sim P$  then complexes with ADP to

yield ATP and free 'X'. The demonstration of  $X\sim P$  should be easier than that of  $X\sim I$ , since it should be possible to feed  $^{32}P_i$  into mitochondria under conditions limiting ATP synthesis and then observe an increase in the label incorporated into the mitochondrial membrane, corresponding to an accumulation of  $X\sim P$ . ATP synthesis could be halted either by depriving the mitochondria of ADP or by blocking phosphorylation with specific inhibitors such as aurovertin.

Boyer<sup>41</sup> has used an ADP-starvation technique to obtain a protein-bound phosphohistidine in mitochondria supplied with  $^{32}P_i$  and substrate. However, these results have been contested since concentrations of oligomycin which caused 95% inhibition of oxidative phosphorylation had no effect on the incorporation of  $^{32}P_i$  into the phosphohistidine. Other attempts to find phosphorylated and non-phosphorylated high energy intermediates are summarized in Table 4.1. It can be seen that none of these intermediates satisfy all the criteria for a high energy intermediate given by Slater.<sup>42</sup>

These criteria are:

- (i) In the presence of mitochondria or mitochondrial extracts it should react with ADP and  $P_i$  (ADP alone for a phosphorylated intermediate), to yield equimolar amounts of ATP.
  - (ii) The formation of the intermediate should be prevented by added dinitrophenol.
  - (iii) Oligomycin should stop the formation of high energy phosphorylated intermediates, or prevent the generation of ATP by added  $P_i$  and ADP in the case of high energy non-phosphorylated intermediates.
- {

(iv)

High energy non-phosphorylated intermediates should contain a respiratory chain component.

}

TABLE 4.1.

PREVIOUSLY REPORTED HIGH ENERGY INTERMEDIATES  
IN ATP SYNTHESIS

Type of Intermediate	'Intermediate'	Comments
Non-phosphorylated	Purvis - 'Extra NAD' High energy complex containing NAD. <sup>43</sup>	No evidence of ATP synthesis
	Pinchot - 'NAD~E' <u>Alcalagenies faecalis</u> extract synthesized ATP. <sup>44</sup>	Contaminating phosphorylase and adenylate kinase enzymes responsible.
Phosphorylated	Brodie - phosphorylated naphthoquinone in Mycob- acterium extracts. <sup>45</sup>	Direct transfer of Pi to ATP not shown.
	Griffths - 'NADH~P' in deproteinized super- natant from mitochon- dria. <sup>46</sup>	Not repeated.
	Boyer - Phosphohistidine labelled in absence of ADP. <sup>41</sup>	Labelling oligomycin insensitive.
	Beyer showed mitochon- drial fraction (CFII) labelled by <sup>32</sup> Pi in presence of substrate. <sup>47</sup>	Involvement of CFII in oxidative phosphorylation not demonstrated.
	Hill et al <sup>48</sup> derived a <sup>32</sup> Pi labelled polar lipid when mitochondria incubated with γ - <sup>32</sup> p - ATP and oligomycin.	No demonstration ATP synthesis, slow labelling rate.

There is now good evidence of phosphorylated protein intermediates in both the microsomal  $\text{Na}^+/\text{K}^+$  ATPase and the  $\text{Ca}^{++}$  ATPase from the sarcoplasmic reticulum. It can be shown using  $\gamma$ - $^{32}\text{P}$ -ATP that there is a labelling of the  $\text{Na}^+/\text{K}^+$  ATPase in the absence of  $\text{K}^+$ . When  $\text{K}^+$  is added to the system the  $^{32}\text{Pi}$  is hydrolysed off the membrane. Various mechanisms have been proposed for the formation of a phosphorylated intermediate in this  $\text{Na}^+/\text{K}^+$  ATPase.<sup>49-51</sup>

The nature of the phosphorylated intermediate has been shown to be an acyl linkage between the terminal Pi of the ATP and a membrane carboxyl group.<sup>52</sup> Kahlenburg et al<sup>53</sup> have since shown that this acyl bond is via the  $\gamma$  carboxyl group of a glutamyl residue in the enzyme. The phosphorylated intermediate may take up two conformations, depending on the ionic environment and the energy level in the membrane.<sup>54,55.</sup>

Martonisi<sup>56</sup> has shown that the  $\text{Ca}^{++}$  dependant sarcoplasmic reticulum ATPase will catalyse an ATP-ADP exchange and that it may be phosphorylated with  $\gamma$ - $^{32}\text{P}$ -ATP. Panet et al<sup>57</sup> concluded that there was a sulphydryl group present at the active site of the enzyme.

#### 4.2. Inhibitors of Oxidative Phosphorylation

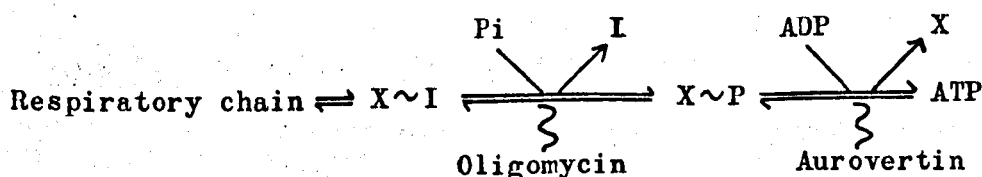
Various specific inhibitors of oxidative phosphorylation have been isolated and by studying their effects on the ATP-synthetic pathway certain conclusions have been drawn as to the relationship of the high energy intermediates to ATP. The three inhibitors of phosphorylation that have been used in this research are DCCD, oligomycin and aurovertin. Since the mode of DCCD inhibition is discussed in great detail in the next chapter it will not be dealt with

here.

Both oligomycin and aurovertin inhibit oxidative phosphorylation by action on the coupling system rather than acting on the respiratory chain. Their modes of action are, however, quite distinct and Robertson et al<sup>20</sup> have reviewed the evidence as to their relative sites of action on the respiratory chain. A distinct difference between the two inhibitors is that while oligomycin has no effect on the solubilized mitochondrial ATPase,  $F_1$ ,<sup>58</sup> aurovertin will still inhibit this soluble form of the ATPase.<sup>59</sup>

Also oligomycin inhibits oxidative phosphorylation with equal efficiency whether the system is utilising or generating ATP, whereas aurovertin is a more potent inhibitor when the system is being driven in the forward, i.e. ATP synthesis, direction rather than when the ATPase is active.<sup>60</sup>

Lardy et al<sup>61</sup> originally proposed that aurovertin acted prior to oligomycin in the ATP generating sequence. However, the current consensus of opinion is that the opposite is correct as the scheme below shows:



Evidence for this scheme is provided by the following observations:

- (1) Oligomycin stimulates the aerobic energy linked pyridine nucleotide transhydrogenase, but aurovertin has no effect on this reaction. A possible explanation for this observation is that oligomycin inhibition causes an accumulation of a high energy intermediate in the absence of ATP synthesis and this intermediate drives the transhydrogenase.

- (2) The effect of oligomycin and aurovertin on arsenate stimulated mitochondrial respiration (see below)
- (3) Mitchell and Moyle<sup>62,131</sup> have shown that aurovertin acts on isolated ATPase by lowering the  $K_m$  for ATP, this implies that aurovertin acts at the terminal ATP synthesis, step.
- (4) Lee and Ernster<sup>63</sup> have shown that oligomycin can <sup>inhibit</sup> the NADH oxidase of 'non-phosphorylating' particles in the absence of  $P_i$ , but aurovertin has no effect under these conditions, i.e. oligomycin is active in the absence of  $X\sim P$ , indicating that it acts prior to the synthesis of  $X\sim P$ .

The results so far support the idea of high energy intermediate(s), but there is no definitive evidence for the existence of a high energy phosphorylated intermediate distinct from a non-phosphorylated intermediate. Indeed the failure to conclusively isolate a phosphorylated intermediate from the many experimental approaches using  $^{32}P_i$  incorporation suggests that there may not be such a phosphorylated intermediate. However, a hypothesis cannot be based on such negative evidence, the explanation for the failure to isolate a high energy phosphorylated intermediate may either be a very low concentration of such a complex within the mitochondrion or the inherent lability of the complex which hydrolyses when isolation is attempted.

#### 4.3. Arsenate and the Respiratory Chain

Arsenate is one of the classical uncoupling agents of oxidative phosphorylation, addition of inorganic phosphate,  $P_i$ , removes the arsenate ( $As_i$ ) effect but by further adding ADP to the system the arsenate uncoupling effect is evident again.



Arsenate stimulated mitochondrial respiration is completely inhibited by oligomycin but aurovertin has a more complex effect.<sup>6</sup> The results obtained with aurovertin have been explained by the following mechanism.<sup>63</sup>

Added arsenate ( $\text{As}_i$ ) competes with inorganic phosphate to form  $\text{X}\sim\text{As}$  instead of  $\text{X}\sim\text{P}$ . The ADP-As formed from  $\text{X}\sim\text{As}$  is unstable and hydrolyses, thus the arsenate is acting as an uncoupling agent and mitochondrial respiration is stimulated. However, in the presence of low concentrations of aurovertin,  $< 0.2 \mu\text{g aurovertin/mg protein}$ , the arsenate-stimulated respiration is inhibited, this is explained by suggesting that the formation of ADP-As from  $\text{X}\sim\text{As}$  is inhibited by the presence of aurovertin. At higher concentrations of aurovertin,  $> 0.5 \mu\text{g aurovertin/mg protein}$  there is no effect on the arsenate stimulated respiration. This observation is explained by proposing that at these high concentrations of aurovertin the  $\text{X}\sim\text{As}$  becomes unstable and hydrolyses, therefore the aurovertin does not inhibit the arsenate stimulated respiration at these higher concentrations.

It appeared that oligomycin acted prior to the involvement of  $\text{P}_i$  or  $\text{As}_i$  since it completely inhibits the arsenate stimulated respiration.

Recently, however, the use of arsenate for comparing the sites of aurovertin and oligomycin action has been questioned. It has now been shown that arsenate acts directly on the mitochondrion by lowering the  $K_m$  for ATP in the ATP-driven reversal reaction. The  $V_{\text{max}}$  was unaltered and Huang and Mitchell<sup>64</sup> suggest that rather than acting as an alternative substrate to  $\text{P}_i$  the  $\text{As}_i$  is primarily an enzyme modifying agent.

It had been shown that there is an  $As_i - H_2^{18}O$  exchange reaction and this had been given as evidence of the involvement of  $As_i$  in high energy intermediates, parallel to the  $Pi - H_2^{18}O$  partial reaction. However, DeMaster and Mitchell<sup>65</sup> have shown that the arsenate exchange is insensitive to both oligomycin and DNP, thus it seems very unlikely that the arsenate reaction can be equated to a partial reaction. Mitchell et al<sup>66</sup> have since shown that the arsenate reaction is also not dependant on the presence of ADP, unlike the  $Pi - H_2^{18}O$  exchange.

Thus although the 'arsenolytic theory' no longer provides such good evidence for the reaction sequence of aurovertin and oligomycin there is still less direct evidence that the sequence of inhibition proposed earlier is correct.

## Results

### 4.4. The Biological and Physical Properties of Aurovertin

Two sources of aurovertin were used in these experiments:

- (1) Lardy aurovertin (L.aurovertin)
- (2) Sittingbourne aurovertin (S.aurovertin)

(1) is a sample of the original aurovertin produced by the Pitman-Moore division of the Dow Chemical Co., having been donated by Prof. Lardy. The second type, S.aurovertin, was extracted from a Calcasporium sp. by D. Farrant at the Sittingbourne laboratories. The extraction procedure that was used is described in Chapter 2.

The initial experiments were designed to determine if S.aurovertin had similar biochemical and physical properties to the original L.aurovertin.

(a) Physical characteristics of the Aurovertins.

Both the S.aurovertin and the L.aurovertin showed absorption maxima at 270, 274 and 370 nm. The two aurovertins ran coincidentally on TLC, using the chloroform-acetone system described earlier.

(b) Biological characteristics of the Aurovertins.

The inhibitory effect of S.aurovertin on the ADP-stimulated respiration of beef heart mitochondria was determined, this respiration being 98.5% inhibited by 0.25  $\mu$ g S.aurovertin/mg. mitochondrial protein. This is identical to the value reported by Robertson et al.<sup>20</sup> The S. and L. aurovertins were also examined for their effect on the energy linked pyridine nucleotide transhydrogenase in beef heart mitochondria, the inhibitory actions of both aurovertins were identical with that shown by Robertson et al.<sup>20</sup>

The complex effect of S.aurovertin on arsenate-stimulated respiration in rat liver mitochondria is illustrated in Figure 4.1. S.aurovertin was titrated against the respiration rate due to arsenate stimulation. The experimental protocol was to obtain coupled rat liver mitochondria, add succinate as a substrate followed by arsenate to obtain a stimulation of respiration, the indicated concentration of S.aurovertin was then added to inhibit this stimulation. Finally phosphate buffer, at pH 7.5, was added to inhibit any remaining arsenate stimulated respiration.

The ordinate in Figure 4.1 represents, in nmoles  $O_2$ /min/mg. protein, the rate of arsenate stimulated respiration in the presence of S. aurovertin minus the basal rate of respiration after the addition of phosphate. It can be seen that at 0.027  $\mu$ g aurovertin/mg. protein the arsenate-stimulated respiration was completely inhibited, but at aurovertin concentrations above 0.14  $\mu$ g aurovertin/mg. protein the inhibitor had a decreasing effect on the arsenate-

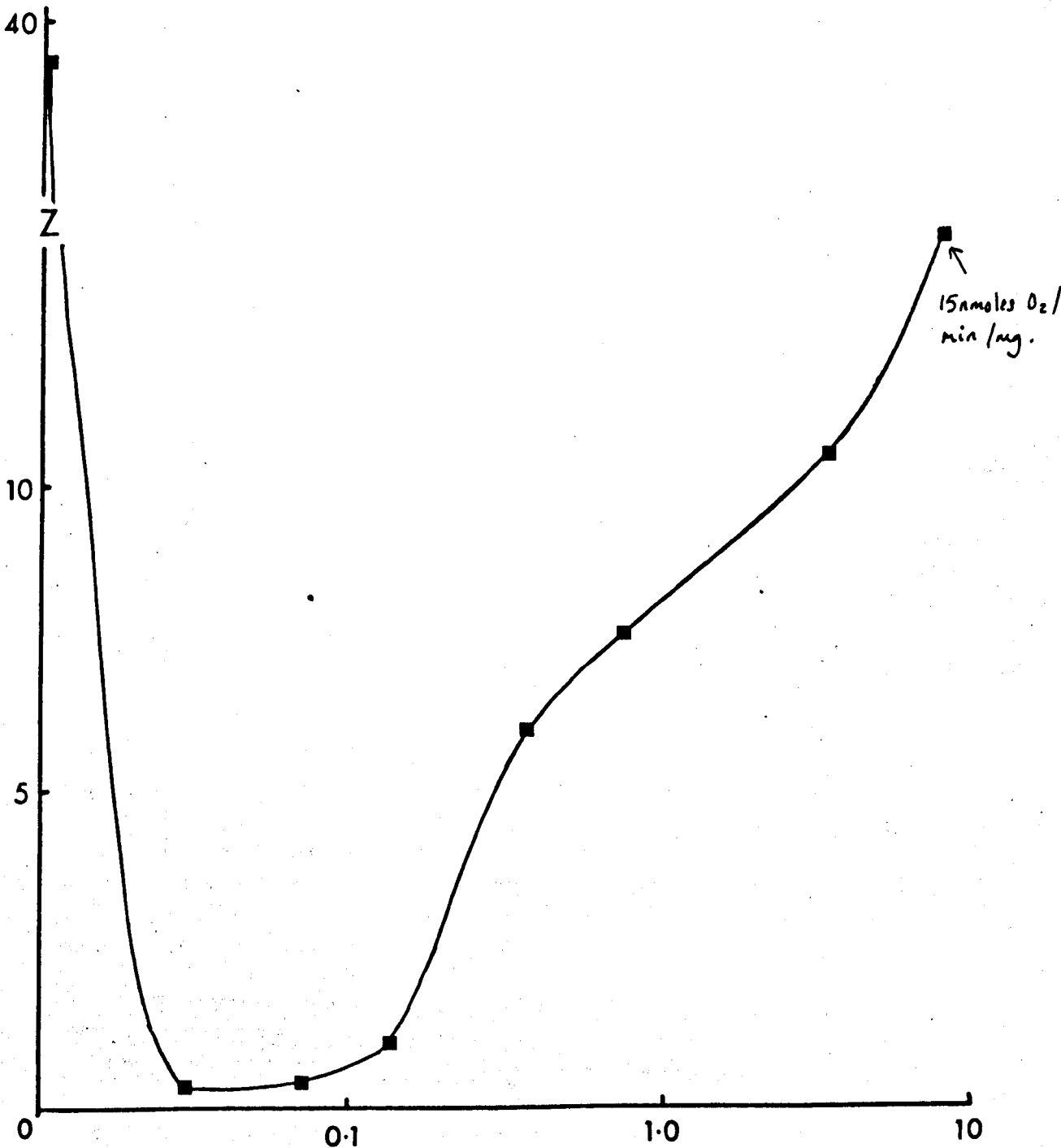
FIGURE 4.1.

S.AUROVERTIN INHIBITION OF THE ARSENATE-STIMULATED  
RESPIRATION IN RAT LIVER MITOCHONDRIA

0.1 ml. of a 50 mg/ml. mitochondrial suspension was added to 3.5 ml. of 15 mM KCL, 50 mM Tris-SO<sub>4</sub> pH 7.5, 1 mM EDTA, 2.5 mM MgCl<sub>2</sub>, 60 mM succinate in a Clarke oxygen electrode chamber at 30°C.  $150 \times 10^{-12}$  moles of rotenone, dissolved in ethanol were then added. When the respiratory rate was linear arsenate was added to a final concentration of 20 mM. 60 seconds later a small volume, <20  $\mu$ l, of S.aurovertin dissolved in ethanol was added to the concentration indicated. After the respiratory rate was linear phosphate buffer at pH 7.5 was then added to a final concentration of 20 mM. The ordinate represents, in nmoles O<sub>2</sub>/min/mg. protein, the arsenate stimulated respiratory rate in the presence of aurovertin minus the rate after the addition of phosphate.

As<sub>i</sub> respiration

( nmoles O<sub>2</sub>/min/mg )



µg *S. aurovertin*/mg mitochondrial protein

stimulated respiration.

Although the overall biphasic effect of S.aurovertin on the arsenate-stimulated respiration was very similar to that reported by Cross and Wang<sup>6</sup> there are detailed differences. The most marked is that S.aurovertin had a maximal inhibitory effect at 0.027  $\mu\text{g}/\text{mg}$ , completely inhibiting the arsenate-stimulated respiration. In comparison Cross and Wang<sup>6</sup> showed a maximal inhibitory effect at 0.15  $\mu\text{g}$  aurovertin/mg, there being little inhibition at 0.03  $\mu\text{g}/\text{mg}$ . At the optimal inhibitory concentration the S.aurovertin completely inhibited the arsenate-stimulated respiration, but Cross and Wang<sup>6</sup> never obtained the complete inhibition of the stimulated respiration.

However, it was concluded that the S.aurovertin was essentially the same as that supplied by the Dow Chemical Co., and therefore was suitable for further experimental use.

The mitochondria used in these experiments were all well coupled. The freshly prepared mitochondria had to show a respiratory control ratio of at least 4 with succinate as a substrate before they were used in the  $^{32}\text{P}_i$  incorporation studies. The mitochondria were prepared immediately prior to each experiment and were not kept for more than 2 hours before use. The details of the preparation of the mitochondria are given in Chapter 2, sections 2.4 and 2.5. The oxygen electrode experimental details are described in section 2.11.

#### 4.5. $^{32}\text{P}_i$ Incorporation Experiments

The initial experiments were a repeat of those described by Cross et al.<sup>7</sup> The experimental protocol for this work is illustrated in Table 4.2. Two mitochondrial suspensions were treated in parallel, one inhibited at 0 seconds by aurovertin, the other by oligomycin. Succinate was added to both suspensions, followed by  $^{32}\text{P}_i$  and

TABLE 4.2.  
PROTOCOL FOR  $^{32}\text{Pi}$  INCORPORATION EXPERIMENTS

A concentrated mitochondrial suspension, (50-60 mg. mitochondrial protein/ml.) was diluted to a final protein concentration of 9 mg/ml. with a solution of 0.25 M sucrose, 20 mM Tris pH 7.4, 2 mM  $\text{MgCl}_2$ , 10 mM KCl. The volume of each sample and control incubation was 2.0 ml. of the 9 mg/ml. suspension. The experiments were performed at 30°C. Aurovertin and oligomycin, both dissolved in ethanol were added, in a minimum volume, <10  $\mu\text{l}$ , to concentrations of 1.2  $\mu\text{g}$  inhibitor/mg. mitochondrial protein. The succinate was added to a final concentration of 7.5 mM, the final concentration of the added carrier Pi plus  $^{32}\text{Pi}$  was 0.1 mM. The perchloric acid (72% w/v), was pre-cooled in a dry ice/acetone bath before adding to the mitochondrial suspension. 15 ml. was added to both the control and sample incubations 120 seconds after the experiment had begun. The protein precipitate was collected by centrifugation at 16,000 g for 10 minutes at 0°C. The resulting pellet was resuspended in 20 ml. of the perchloric acid, (72% w/v), and washed again. The washing was repeated once more, then the final precipitate was resuspended in 0.5 ml. of distilled water and this suspension was transferred to a scintillation vial, containing 10 ml. of the Triton-Toluene-BPD scintillant described previously. Another 0.2 ml. of distilled water was used for washing the centrifuge tubes. This was also transferred to the scintillation vial. The radioactivity in each vial was normally measured for 10 minute periods.

Time (Seconds)	Additions	
	Sample	Control
0	Aurovertin	Oligomycin
20	Succinate	Succinate
40	Carrier Pi plus $^{32}\text{Pi}$	Carrier Pi plus $^{32}\text{Pi}$
100	Oligomycin	Aurovertin
120	72% (w/v) Perchloric Acid	72% (w/v) Perchloric Acid



carrier Pi. After a further 40 seconds incubation the mitochondrial suspension was precipitated by treatment with perchloric acid. The resulting pellet was washed and the radioactivity incorporated into the protein precipitate was measured. In the experiments of Cross et al<sup>6</sup> there is a greater incorporation of radioactivity into the sample, aurovertin treated, mitochondria and it is suggested that this differential incorporation is due to the accumulation of a high energy phosphorylated intermediate.

There were various details in the experimental procedure that were different from the procedure described by Cross et al<sup>6</sup>. These are listed below:

- (1) Carrier Pi and  $^{32}\text{Pi}$  were added together and not as separate solutions as described by Cross et al<sup>6</sup>.
- (2) The perchloric acid precipitate was washed by vortex mixing rather than by disturbing the pellets with glass rods.
- (3) Rotenone was omitted from the incubation medium in the later experiments, but this had no effect on the results.

The results of the first series of experiments are shown in Table 4.3. Two separate sets of incubations were performed, each consisting of three control and three sample experiments.

For the first set of experiments the average total cpm in the washed PCA precipitate in the control experiments was  $1571 \pm 304$ , (95% confidence limits being shown), the average total cpm in the sample experiments was  $2465 \pm 914$ . These results give an average excess incorporation of 0.034 nmoles Pi/mg protein, expressed in terms of the final protein present in the washed PCA precipitate, into the sample incubations.

TABLE 4.3.

FIRST SERIES OF DIFFERENTIAL  $^{32}\text{Pi}$   
INCORPORATION EXPERIMENTS

The experiments were performed at  $30^{\circ}\text{C}$  as described in Table 4.2, with succinate as a substrate, using rat liver mitochondria. The first set of three results shown were obtained using a final volume of 2.0 ml of the 9 mg/ml mitochondrial suspension.  $2 \times 10^6$  cpm of  $^{32}\text{Pi}$  was added to each incubation in the first set of results. The final concentration of added Pi was 0.1 mM. The second set of three results are from incubations which were of a final volume of 0.8 ml of the 9 mg/ml mitochondrial suspension  $8 \times 10^6$  cpm of  $^{32}\text{Pi}$  was added to each incubation in this set of experiments. The total added Pi +  $^{32}\text{Pi}$  was still a final concentration of 0.1 mM. This gave a final total concentration, added plus endogenous, of 0.55 mM for each incubation. Lardy aurovertin was used for all these experiments.

Endogenous Pi concentration in mitochondria (mM)	cpm in washed PCA precipitate		% change in sample	$\Delta$ nmole Pi/mg. protein
	Sample	Control		
0.45	2055	1734	+ 27.5 )	0.034
	2782	1261	+ 120.0 )	
	2559	1718	+ 47.7 )	
0.45	9294	7688	+ 19.6 )	0.026
	10938	9072	+ 20.6 )	
	9101	7211	+ 26.6 )	

The second set of experiments gave an average total cpm for the washed PCA precipitate in the control experiments of  $7990 \pm 911$  and for the sample experiments  $9771 \pm 951$ .

The average excess incorporation of  $^{32}\text{Pi}$  for this set of experiments was 0.026 nmoles Pi/mg protein. These values compare with the excess incorporation of 0.05 nmoles Pi/mg protein reported by Cross et al.<sup>6</sup>

Following the initial success at repeating the experiments further work, using the same experimental technique, was performed at the Warwick and Sittingbourne laboratories. These experiments used both L.aurovertin and S.aurovertin and the results are summarized in Table 4.4. In order to conserve the aurovertin the experiments were scaled down from a final volume of the mitochondrial suspension 2.0 ml. to either 0.8 ml. or 0.4 ml.

These results consistently failed to reproduce the initial observations, there being no significantly greater  $^{32}\text{Pi}$  incorporation in the sample mitochondria. The experiments were performed with both beef heart and rat liver mitochondria, also various concentrations of  $^{32}\text{Pi}$  were added to the carrier phosphate, accounting for the variation in  $^{32}\text{Pi}$  incorporated into the protein precipitates.

The failure to repeat the initial observations of a differential  $^{32}\text{Pi}$  incorporation into the sample and control mitochondria was not thought to be due to reducing the volume of the mitochondrial suspension. The variation in the radioactivity incorporated into the PCA precipitates in the second series of experiments was not greater than that seen in the first series as shown by Tables 4.3. and 4.4. Also one set of results in the first series of experiments, Table 4.3, had been obtained using a small volume of the mitochondrial suspension.

TABLE 4.4.

SECOND SERIES OF DIFFERENTIAL  $^{32}\text{Pi}$   
INCORPORATION EXPERIMENTS

These incubations were performed as described in Table 4.2. Both beef heart and rat liver mitochondria were used for the incubations, as indicated in the table.

The final volume of 9 mg/ml. mitochondrial suspension was either 0.8 ml. or 0.4 ml.

Aurovertin and oligomycin were both added to a final concentration of 1.2  $\mu\text{g}$  inhibitor/mg. mitochondrial protein. In the final set of four results, \* , the succinate solution was added to the mitochondrial suspension 80 seconds after the first inhibitor had been added, the phosphate was then added at 100 seconds, the second inhibitor at 160 seconds and finally the perchloric acid at 180 seconds.

The total  $^{32}\text{Pi}$  associated with the washed PCA precipitate was measured for 10 minute periods. The  $^{32}\text{Pi}$  added to each incubation varied with each set of results. The upper and lower limits of added  $^{32}\text{Pi}$  were  $3.5. \times 10^8$  and  $6.3. \times 10^5$  cpm. The total added ( $\text{Pi} + ^{32}\text{Pi}$ ) was always to a final concentration of 0.1 mM.

Mitochondria	Type of aurovertin	cpm in washed PCA precipitate		% change in sample
		Sample	Control	
BIM	S	4190	4075	+ 2.8
		4224	4701	- 10.1
		4332	4426	- 2.1
BIM	S	16736	15152	+ 9.4
		14887	13401	+ 9.9
		14153	15053	- 6.3
BIM	L	4694	5051	- 7.0
		4422	4632	- 5.0
		5282	4024	+ 14.0
BIM	S	9494	9932	- 4.4
		10869	9745	+ 11.5
		10244	10100	+ 4.4
RLM	L	3634	3770	- 3.6
		3805	3772	+ 0.8
		3490	3665	- 4.7
RLM	S	9714	9996	- 2.8
		10199	9678	+ 4.7
		9908	9757	+ 1.5
		9183	9670	- 5.0
RLM *	L	4862	4709	+ 3.0
		4442	4785	- 7.0
		4746	4450	+ 7.0
		4099	4429	- 7.0

To ensure that the initial inhibitor had been given sufficient time to interact with the mitochondria the aurovertin and oligomycin solutions were added to the sample and control mitochondria 80 seconds before the succinate in the final set of experiments shown in Table 4.4. However, this made no difference to the relative  $^{32}\text{Pi}$  incorporations seen in the sample and control mitochondria.

In an attempt to resolve these inconsistencies the various parameters affecting the  $^{32}\text{Pi}$  incorporation were investigated. Two possible reasons for the lack of differential  $^{32}\text{Pi}$  incorporation were:

(a) A 'leak' around the oligomycin inhibition in the control mitochondria, allowing  $\text{X}\sim\text{P}$  to accumulate.

(b) A 'leak' around the aurovertin inhibition in the sample mitochondria, allowing the discharge of  $\text{X}\sim\text{P}$ .

The first possibility was checked by titrating the oligomycin concentration in the 'control' experiments and determining the concentrations of  $^{32}\text{Pi}$  incorporated into the PCA precipitates. The results of this experiment are given in Table 4.5, which shows that above  $0.5\text{ }\mu\text{g}$  oligomycin/mg mitochondrial protein there is a decrease in the  $^{32}\text{Pi}$  incorporated into the PCA protein precipitate. It appeared, therefore, that  $^{32}\text{Pi}$  incorporation in the control mitochondria was at least being partially inhibited by the oligomycin. No further decrease in the  $^{32}\text{Pi}$  incorporation was observed at oligomycin concentrations up to  $20\text{ }\mu\text{g}/\text{mg}$  protein, therefore,  $1.2\text{ }\mu\text{g}$  oligomycin/mg protein should produce maximum inhibition of the mitochondria.

Mitochondria were then titrated with aurovertin, using a similar procedure to the previous experiment except that the sample was

TABLE 4.5.

TITRATION OF THE OLIGOMYCIN CONCENTRATION  
AND  $^{32}\text{Pi}$  INCORPORATION INTO THE WASHED PCA PRECIPITATE

The experiments were performed using 0.4 ml. of the 9.0 mg/ml. rat liver mitochondrial suspension for each incubation, as described in the legend to Table 4.2. 1.2  $\mu\text{g}$  S.aurovertin/mg protein was used, the concentration of oligomycin being given in the table. An extended time scale was used for this experiment. At 0 seconds the first inhibitor was added to the suspension aurovertin for the sample and oligomycin for the control incubation. At 60 seconds succinate was added to a final concentration of 7.5 mM. At 80 seconds  $5.7 \times 10^5$  cpm of  $^{32}\text{Pi}$  plus carrier phosphate was added to give a final concentration of 0.1 mM added ( $^{32}\text{Pi}$  + Pi). At 140 seconds the second inhibitor, oligomycin in the sample and aurovertin in the control incubation, was added. Finally at 160 seconds 10 ml. of perchloric acid (72% w/w) was added to each incubation. The PCA precipitates were washed and the radioactivity measured as described in the legend to Table 4.2.



$\mu\text{g}$ oligomycin/mg mitochondrial protein	total cpm in washed PCA precipitate	% change with respect to 0.0 $\mu\text{g}$ oligomycin/ mg protein
* 0.0	3822	-
* 0.2	3925	+ 2.6
* 0.5	3079	- 19.4
* 1.0	3130	- 18.1
* 5.0	3260	- 14.7
* 10.0	4447	+ 16.3
* 20.0	3202	- 16.2
+ 5.0	3194	- 16.4

\* Control protocol

+ Sample protocol

titrated with increasing concentrations of aurovertin. The results of this experiment in Table 4.6 show that, compared to the control with no aurovertin, there is no consistent increase in the  $^{32}\text{Pi}$  incorporated into the sample mitochondria with increasing aurovertin concentration.

These experiments showed that, while oligomycin appears to inhibit the  $^{32}\text{Pi}$  incorporation into the PCA precipitate, aurovertin does not cause any differential incorporation of  $^{32}\text{Pi}$  compared to the oligomycin inhibited mitochondria.

A possible explanation for this behaviour was that the mitochondria were deficient in phosphate, and that the accumulation of the phosphorylated intermediate was limited.

This was suggested by the observation that the respiratory rate of state 3 mitochondria could be further increased by adding Pi to a final concentration of 4 mM. This was significantly higher than the endogenous Pi concentration of the rat liver mitochondria used in the second series of experiments, see Table 4.4, which had been found to be 0.1 - 0.2 mM. The endogenous Pi concentration refers to the inorganic phosphate present in the protein-free PCA supernatant after the mitochondrial protein had been precipitated by perchloric acid. The Pi concentration is expressed in terms of the 9 mg/ml. mitochondrial suspension used in the incorporation experiments, i.e., an endogenous Pi concentration of 0.1 mM is equivalent to 11.1 nmoles Pi/mg mitochondrial protein.

The final concentration of Pi in the mitochondrial suspension used in this second series of incorporation experiments was therefore less than 0.3 mM.

To test this parameter a series of differential incorporation

TABLE 4.6.

TITRATION OF THE AUROVERTIN CONCENTRATION  
AND  $^{32}\text{P}$ i INCORPORATION INTO THE WASHED PCA PRECIPITATE

The experiments were performed using 0.4 ml. of the 9.0 mg/ml rat liver mitochondrial suspension for each incubation as described in the legend to Table 4.2. 1.2  $\mu\text{g}$  oligomycin/mg mitochondrial protein was added to each incubation at the appropriate time. The concentrations of aurovertin used are given in the table. The time scale of the experiment is that as described in the legend to Table 4.5.  $5.7 \times 10^5$  cpm of  $^{32}\text{P}$ i was added to each incubation at 80 seconds. The washing of the PCA precipitate and measurement of the radioactivity was performed as described in the legend to Table 4.2.

$\mu\text{g}$ S.aurovertin/mg. mitochondrial protein	total cpm in washed PCA precipitate	% change with respect to 0.0 $\mu\text{g}$ aurovertin/ mg protein
+ 0.0	3164	-
+ 0.05	3216	+ 1.6.
+ 0.1	2797	- 11.6.
+ 0.5	3168	+ 0.1.
+ 1.0	3349	+ 5.5.
+ 5.0	2696	- 14.0.
* 1.0	3005	- 5.0.

\* Control protocol

+ Sample protocol

TABLE 4.7.

THE EFFECT OF INCREASING THE TOTAL Pi  
CONCENTRATION ON THE INCORPORATION OF  $^{32}\text{Pi}$   
INTO THE PCA PRECIPITATES

The experiment was performed at 30°C with rat liver mitochondria, using the protocol described in Table 4.2, except that the concentration of added ( $^{32}\text{Pi} + \text{Pi}$ ) was varied between each pair of sample and control incubations. The final concentration of added ( $^{32}\text{Pi} + \text{Pi}$ ) is shown, together with the total Pi incorporated into the washed PCA precipitates. The PCA precipitates were washed three times with perchloric acid (72% w/w) and then the radioactivity in the washed precipitate was measured as described in the legend to Table 4.2. The aurovertin and oligomycin concentrations used in each incubation were 1.2  $\mu\text{g}$  inhibitor/mg mitochondrial.

* Final concentration of added (Pi + $^3\text{P}$ -Pi) (mM)	Total Pi incorporated into washed PCA precipitate (nmole Pi/ $\mu\text{g}$ protein)		$\Delta$ Pi incorporated into the sample (nmole Pi/ $\mu\text{g}$ protein)
	Sample	Control	
0.1	0.21	0.19	+ 0.02
0.4	0.47	0.60	- 0.13
1.0	0.83	0.84	- 0.01
2.0	1.56	1.56	0.0
4.0	3.62	3.04	+ 0.58

\* Endogenous mitochondrial Pi concentration was 0.12 mM.

experiments were performed, using increasing concentrations of added Pi. The results are summarized in Table 4.7 and they show that, apart from the result at the 4.0 mM added phosphate concentration there was no significant differential incorporation of  $^{32}\text{Pi}$  into the sample mitochondria.

Since this final result at 4.0 mM added (Pi +  $^{32}\text{Pi}$ ) was an isolated observation, not supported by an overall trend in the other results, and also since the differential  $^{32}\text{Pi}$  incorporation was ten times that reported by Cross et al<sup>6</sup> it was thought to be due to experimental error, rather than a significant observation.

Other factors which may have affected the action of aurovertin were tested by K. J. Cattell and D. Farrant at the Sittingbourne laboratories. These included the use of glass centrifuge tubes instead of the standard polypropylene type and also the preparation of rat liver mitochondria using the conditions described by Cross.<sup>67</sup>

These mitochondria exhibited both high respiratory control ratios and  $\text{ADP}/0$  ratios, but the basic observation on differential  $^{32}\text{Pi}$  incorporation was not detected with any of the conditions used.

#### 4.6. Discussion and Conclusions

Apart from the initial series of experiments the results of Cross et al<sup>7</sup> have not been satisfactorily repeated. No obvious explanation has been found for this inconsistency. Recently Cross has found only a 60% success rate in obtaining differential  $^{32}\text{Pi}$  incorporation and he has also shown that the presence of aurovertin is not necessary to obtain a differential incorporation in the sample mitochondria. (P. D. Boyer, personal communication via R. B. Beechey).

This last observation has been repeated, i.e., oligomycin

above a concentration of 0.5  $\mu\text{g}$  oligomycin/mg mitochondrial protein was shown to cause a 16% decrease in  $^{32}\text{P}$ i incorporation compared to untreated mitochondria, cf Table 4.5.

The lack of reproducibility in the incorporation experiments is difficult to explain, since aurovertin had consistent inhibitory effects on energy-linked reactions in mitochondria, e.g. mitochondrial respiration as shown in the oxygen electrode and the ATP driven pyridine nucleotide transhydrogenase reaction.

A possible explanation for the lack of differential  $^{32}\text{P}$ i incorporation in aurovertin inhibited mitochondria, compared to the oligomycin control, could be the instability of the high energy phosphorylated intermediate which hydrolyses rather than accumulates due to aurovertin inhibition. However, this is not valid. If it was correct the aurovertin would act as an uncoupling agent, rather than an inhibitor of mitochondrial respiration.

That oligomycin and aurovertin do not act at the same site seems certain, but the proposition that oligomycin and aurovertin act sequentially before and after the formation of  $X\sim\text{P}$  is not certain. The evidence cited by Cross and Wang<sup>6</sup> depends largely on the effect of aurovertin on arsenate-stimulated respiration in mitochondria. As explained earlier this evidence is now in doubt, since arsenate probably acts primarily as an enzyme modifier rather than replacing phosphate in the energy metabolism.

Other evidence has been provided by Robertson et al<sup>20</sup> using the aerobic driven pyridine nucleotide transhydrogenase reaction which is stimulated by oligomycin, but on which aurovertin has no effect. Since it is proposed that this reaction is driven by  $X\sim\text{I}$  they imply that oligomycin acts nearer  $X\sim\text{I}$  than does aurovertin.



However, this does not demonstrate that oligomycin and aurovertin act on either side of  $X\sim P$ , merely that aurovertin acts nearer the terminal ATP synthesis step than oligomycin.

Thus a possible explanation for the lack of excess  $^{32}P_i$  incorporation in aurovertin inhibited mitochondria is that the aurovertin does not act after the formation of  $X\sim P$ , but prior to its synthesis. This hypothesis is supported by the observations that although oligomycin causes a 16% decrease in the  $^{32}P_i$  incorporated into the PCA pellet, compared to untreated mitochondria, cf Table 4.5, no difference was seen in other experiments in which the mitochondria were inhibited by oligomycin and aurovertin. Therefore, it appeared that aurovertin also had inhibited the formation of  $X\sim P$ .

An alternative to this proposal is that the concentration of  $X\sim P$  in aurovertin treated mitochondria may fluctuate between various mitochondrial preparations, and that the accumulated  $X\sim P$  may be below that detectable with this experimental technique. However, the lack of reproducibility in the results may be due to the mode of aurovertin inhibition on mitochondrial respiration. It has been shown that aurovertin inhibits respiration by lowering the  $K_m$  for ATP in the ATPase complex.<sup>62</sup> This observation implies a rearrangement of the enzyme conformation, and this rearrangement may be sensitive to fine changes in the mitochondrial matrix. Thus the accumulation of  $^{32}P_i$  in the aurovertin-inhibited mitochondria may be a by-product of the primary inhibition and dependant on the final conformation of the mitochondrial membrane.

This hypothesis is supported by the observation that the ADP-stimulated respiration in mitochondria can be routinely inhibited by aurovertin, but the differential  $^{32}P_i$  incorporation is variable.

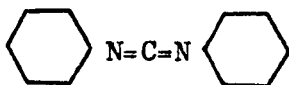
However, use of the method as published by Cross, Cross and Wang<sup>6,7</sup> for further study of the yeast mutants has little application until (a) the results can be routinely demonstrated using mammalian mitochondria, and (b) an explanation can be found for the inconsistent results presented here.

## CHAPTER 5

### THE INTERACTION OF DICYCLOHEXYLCARBODI-IMIDE WITH YEAST SUB-MITOCHONDRIAL PARTICLES

#### Introduction

Dicyclohexylcarbodi-imide, DCCD, is a member of a reactive group of compounds, the carbodi-imides.<sup>68</sup> Because of the reactivity of this group the carbodi-imides have been used as carboxyl activating agents in peptide synthesis.



DCCD, (I)

DCCD is very insoluble in water, but other members of the group such as 1-ethyl-3-(3-dimethyl aminopropyl) carbodi-imide, EDAC, are readily water soluble. This property has a fundamental influence on the interaction of carbodi-imides with biological membranes which have hydrophobic internal environments. This is illustrated by the  $K_i$  values for the inhibition of NADH oxidation in beef heart SMP. The  $K_i$  for EDAC inhibition is 64  $\mu\text{mole/mg}$  protein, that for DCCD is 0.16  $\mu\text{mole/mg}$ .<sup>130</sup>

#### 5.1. DCCD Interactions with mitochondrial membranes

The use of DCCD for investigating oxidative phosphorylation was first introduced by Beechey et al.<sup>69</sup> They demonstrated that DCCD inhibited coupled respiration and ATP-driven partial reactions in Electron Transport Particles from beef heart mitochondria. In a

later reference<sup>10</sup> using ( $^{14}\text{C}$ ) - DCCD it was shown that DCCD binds covalently to beef heart SMP and that the bound ( $^{14}\text{C}$ ) - DCCD could not be removed by washing with either 0.25 M sucrose or iso-octane. The ( $^{14}\text{C}$ ) - DCCD bound to the SMP at the same concentrations found to inhibit the SMP ATPase activity.

Beechey et al<sup>8</sup> have demonstrated that oligomycin and DCCD have similar inhibitory effects on mitochondrial energy-linked reactions. Both oligomycin and DCCD were found to inhibit:

- (1) Coupled mitochondrial respiration, the inhibition being unaffected by arsenate.
- (2) The uncoupler-stimulated ATPase activity of mitochondria and SMP.
- (3) The  $^{32}\text{Pi}$  - ATP exchange in mitochondria.
- (4) The ATP - driven reduction of  $\text{NAD}^+$  by either succinate or ascorbate + TMPD.
- (5) The ATP - driven pyridine nucleotide transhydrogenase.

Minor differences were seen in the action of the two compounds. These can be summarized as:

- (1) The inhibitory effects of oligomycin were immediate, whereas optimal DCCD inhibition required a pre-incubation of up to 24 hours, depending on the temperature and pH of this pre-incubation, and also the concentration of DCCD being used.
- (2) At sub-inhibitory concentrations oligomycin was more efficient than DCCD in stimulating the ATP - driven reduction of  $\text{NAD}^+$  by succinate.
- (3) The inhibition of ATPase activity in DCCD treated SMP cannot be relieved by washing with phospholipids, unlike the reversible oligomycin inhibition.

To date no other significant differences have been reported in the actions of DCCD and oligomycin on energy-linked reactions in

mitochondria. Using beef heart SMP labelled with ( $^{14}\text{C}$ ) - DCCD Cattell and his colleagues have been able to show that 90% of the ( $^{14}\text{C}$ ) - DCCD bound to the SMP is extractable by chloroform-methanol, and that this extract may be further purified by ether precipitation and elution of the redissolved ether precipitate from a Sephadex LH - 20 column.<sup>70</sup> The procedure gives a single DCCD-binding protein which has a 10,000 M.W. by SDS polyacrylamide gel electrophoresis.<sup>70</sup> Recent work using amino acid analysis techniques gives a more accurate estimation of 13,500 for the M.W. of this DCCD binding protein, (K. J. Cattell, personal communication).

Recently Stekhoven et al<sup>71,72</sup> have used a soluble oligomycin sensitive ATPase complex prepared from beef heart mitochondria to demonstrate the existence of the DCCD binding protein.

They have shown that a specific protein, M.W. 13,000 - 14,000, binds DCCD within this OS ATPase complex at the same concentrations that are necessary to inhibit the ATPase activity.

Stekhoven et al showed that using ( $^{14}\text{C}$ ) - DCCD at concentrations above that necessary to inhibit the ATPase activity, i.e., greater than 5 nmoles DCCD/mg protein, other proteins in the OS ATPase complex were labelled, including the  $\text{F}_1$  subunits.<sup>72</sup>

## 5.2. Bacterial Membrane Studies Using DCCD

The cell membrane ATPase from Streptococcus faecalis is also inhibited by DCCD, but unlike the mitochondrial ATPase activity which is involved in oxidative phosphorylation this bacterial ATPase is concerned with selective ion transport, S. faecalis deriving energy primarily by glycolysis.<sup>73</sup>

Harold et al<sup>74</sup> have demonstrated that by inhibiting this cell membrane ATPase DCCD stops  $\text{K}^+$  transport across the membrane, but

this inhibition does not result in a gross disarrangement of the membrane. Abrams and Harold<sup>75</sup> have also shown that release of the ATPase complex from DCCD - treated membranes restores ATPase activity, implying that the primary site of DCCD action is on the membrane rather than the ATPase enzyme itself.

It was also shown that water soluble carbodi-imides have a lower efficiency in inhibiting the membrane bound ATPase, suggesting that the site of DCCD action is within the lipid matrix of the membrane. Abrams et al<sup>76</sup> have now isolated a 'Carbodi-imide Sensitivity Factor', CSF, from the S.faecalis cell membrane which is necessary for DCCD sensitivity. The use of a mutant which is 100-fold less sensitive to DCCD<sup>76</sup> may further elucidate the role of the CSF.

Binding studies with (<sup>14</sup>C) - DCCD and S.faecalis cell membranes have also been performed at the Sittingbourne laboratories.<sup>77</sup> It was found that a maximal 60% inhibition of the ATPase activity could be achieved with 1  $\mu$ mole DCCD/mg protein. 1 nmole DCCD/mg protein gave 45% inhibition of the ATPase activity. If (<sup>14</sup>C) - DCCD was applied to the membranes at 1 nmole DCCD/mg protein and the membranes washed with 0.25 M sucrose only 0.25 nmole DCCD/mg protein remained attached to the membrane.

This apparent inconsistency between the inhibitory concentrations and the amount of (<sup>14</sup>C) - DCCD bound to the cell membranes will be referred to later, in connection with the interaction of DCCD and yeast SMP. No evidence was found of a protein - DCCD complex being formed on the S.faecalis membranes.

It has been shown, however, that DCCD can form a stable covalent bond with phosphatidyl serine.<sup>78</sup>

### 5.3. Mechanism of Carbodi-imide Inhibition

DCCD and oligomycin have very similar effects on energy linked functions in mitochondria, there being no partial reaction that one inhibitor affects exclusively. At present the evidence suggests that both DCCD and oligomycin act by preventing the formation of a high energy phosphorylated intermediate from a high energy non-phosphorylated intermediate.<sup>8</sup>

Further evidence on the mode of carbodi-imide inhibition has been provided by Godin and Schrier<sup>79</sup> who investigated the inhibition of red blood cell ATPase activity by the carbodi-imide EDAC. Because of the water soluble nature of EDAC higher concentrations, compared to DCCD, were necessary to inhibit the ATPase activity. The  $K_m$  of the ATPase was unaltered, but the  $V_{max}$  was lowered by the addition of EDAC to the membranes. This may be compared with the action of aurovertin which lowers the  $K_m$  of the ATPase for ATP, but leaves the  $V_{max}$  unaltered.<sup>62</sup> It was also found that addition of the nucleophile glycine ethyl ester, (GEE), prior to the EDAC, protected the membrane ATPase from EDAC inhibition. Using ( $^{14}C$ ) - GEE it was shown that under these conditions there was an EDAC - dependant binding of the ( $^{14}C$ ) - GEE to the membranes.

The protective effect of GEF was found to be shared by other nucleophiles, such as hydroxylamine, the most effective protective agents being the more lipophilic nucleophiles.

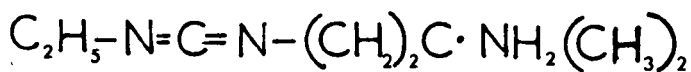
No firm conclusion was reached as to the group on the membrane with which EDAC was reacting, but it was thought that a carboxyl was the most likely.

Their results<sup>79</sup> suggest that the mode of EDAC inhibition and GEE protection was as shown in Figure 5.1. and explained below.

FIGURE 5.1.

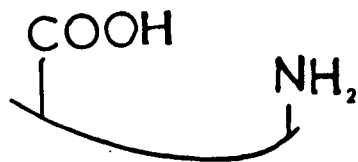
PROPOSED MECHANISM OF EDAC INHIBITION OF  
RED BLOOD CELL ATPase AND PROTECTION BY GEE



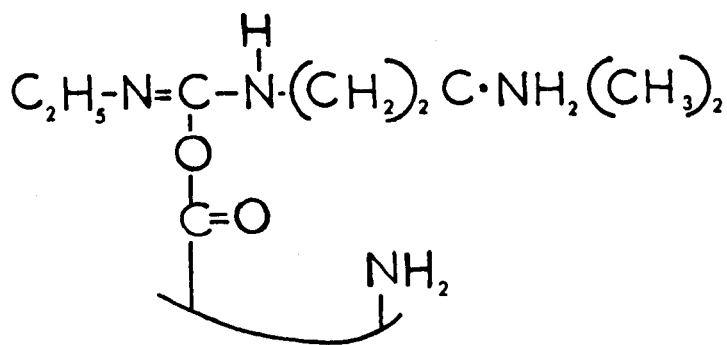


EDAC (II)

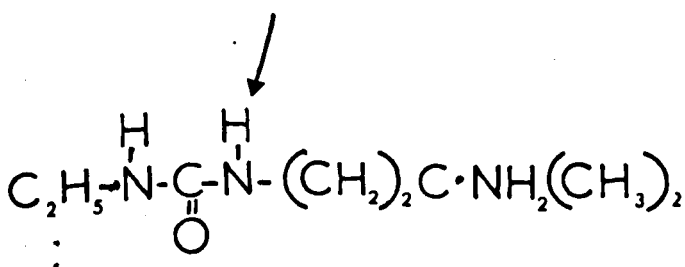
+



Red blood cell  
membrane



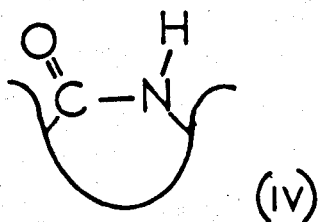
EDAC-activated  
carboxyl (III)



1-ethyl-3-(3 dimethylaminopropyl)urea

(V) , EDAU

+



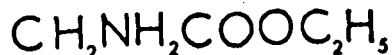
(IV)

Intramembrane bond

ATPase inhibited

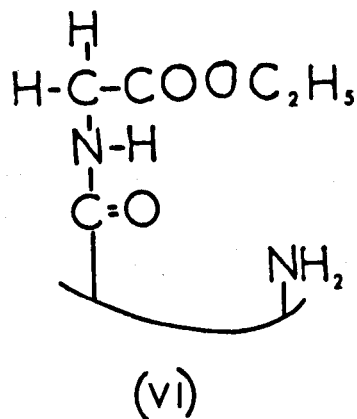
PLUS

Glycine ethyl ester



EDAU (V)

+



(VI)

ATPase active

(1) EDAC, (II) reacts with a membrane group, in this example a carboxyl is shown, to form an activated complex (III).

(2) In the absence of an added nucleophile this activated carboxyl then reacts with a neighbouring membrane nucleophile group, represented here by an amino group, to form a stable intramembrane peptide bond, (IV) and liberating free 1 - ethyl - 3 - (3 - diaminopropyl) urea (V).

(3) However in the presence of GEE, this competes with the membrane nucleophile and preferentially reacts with the activated carboxyl, (III), forming (VI), the free urea being liberated again.

Inhibition by EDAC is therefore due to the formation of the intramembrane bond, (IV) which may cause a membrane rearrangement that is unfavourable to enzyme action. The GEE prevents the formation of this intramembrane bond, stopping the EDAC inhibition of ATPase activity.

:

## Results

### 5.4. DCCD Inhibition of yeast SMP ATPase

The inhibition, by DCCD, of SMP ATPase activity is shown in Figure 5.2, SMP from strains D22 WT, D22 A16 and D22 A21 being used. It can be seen that although both D22 A16 and D22 A21 are resistant to DCCD, compared to D22 WT, the strain D22 A21 is also much more resistant than D22 A16, as measured by the sensitivity of the SMP ATPase activity. This result is quite significant since the strains D22 A16 and D22 A21 have identical sensitivities to oligomycin, and this provides one of the few examples of a differential effect of oligomycin and DCCD on mitochondrial functions. Various other

FIGURE 5.2.

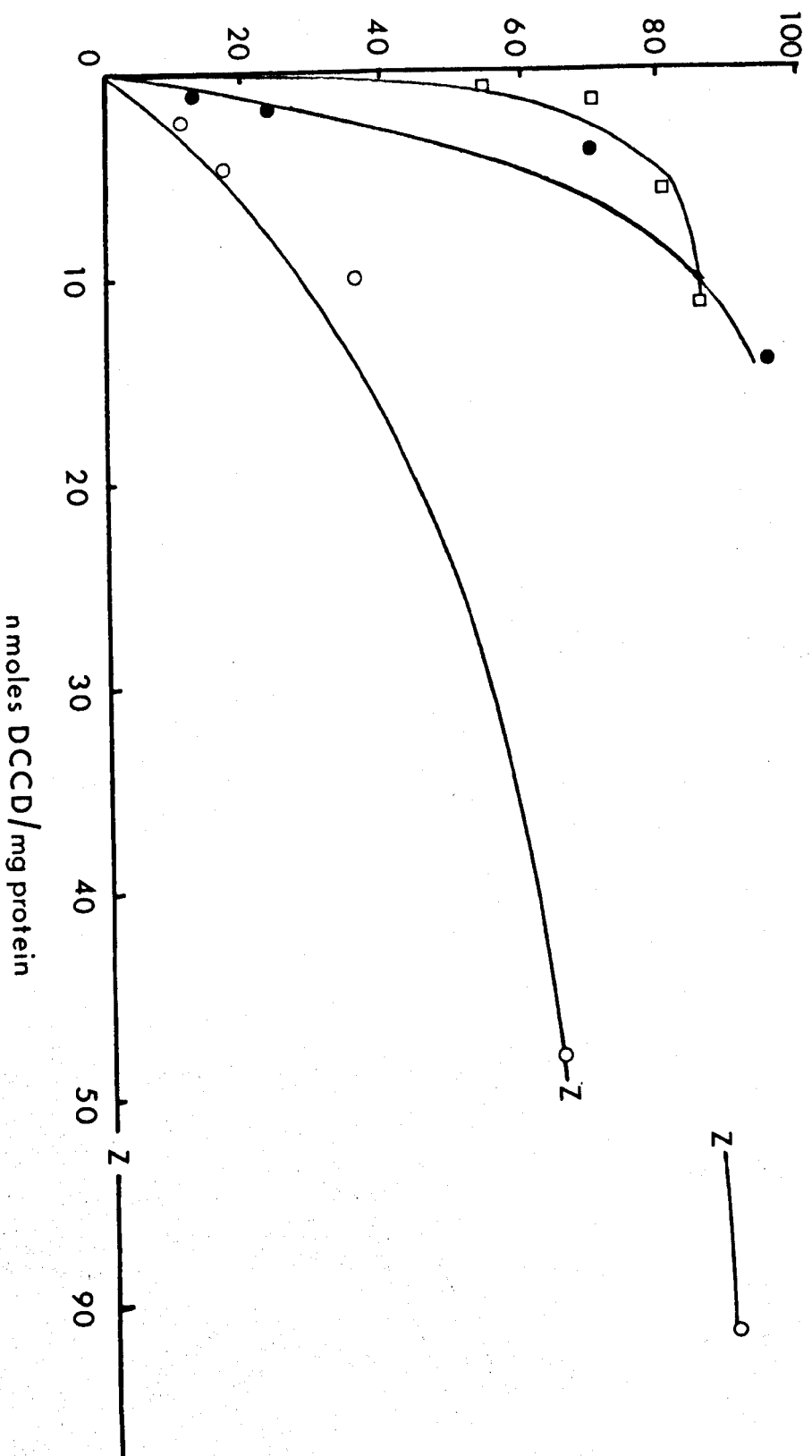
DCCD INHIBITION CURVES OF SMP ATPase ACTIVITY

SMP at a protein concentration of 20 mg/ml in 0.25 M sucrose, 0.1 M Tris - Cl pH 7.5 were incubated with DCCD at the indicated concentrations for 4°C x 16 hours. The DCCD was added in a small volume, < 10 µl, in dimethyl formamide. The ATPase activity was measured at 30°C and pH 9.5 using the NADH oxidation method described in Chapter 2, section 2.10. There was no washing of the SMP before measurement of the ATPase activity.

- D22 WT, ATPase activity : 0.6 µmoles/min/mg
- D22 A16, ATPase activity : 0.9 µmoles/min/mg
- D22 A21, ATPase activity : 1.4 µmoles/min/mg.

% Inhibition of

ATPase



yeast mutants originally isolated by P. R. Avner<sup>1</sup> also show differential resistances between oligomycin and DCCD.<sup>3</sup>

However, since DCCD does inhibit the yeast SMP ATPase activity and its inhibitory effects are very similar to oligomycin the use of (<sup>14</sup>C) - DCCD may well give some information on the nature of resistance to oligomycin and DCCD seen with certain yeast mutants.

Preliminary work by P. R. Avner and D. R. Peers<sup>80</sup> had shown that (<sup>14</sup>C) - DCCD only bound to yeast mitochondria at concentrations much lower than those found necessary to inhibit the SMP ATPase activity. This is quite distinct from the behaviour of (<sup>14</sup>C) - DCCD and beef heart mitochondria, where the DCCD binds to the membranes at the same concentrations required to inhibit the ATPase activity.<sup>10</sup> The results presented here are both an investigation into the use of (<sup>14</sup>C) - DCCD for studies on the yeast mutants and also experiments designed to resolve the apparent inconsistency between the amount of (<sup>14</sup>C) - DCCD bound to yeast SMP and the concentration of DCCD necessary to inhibit SMP ATPase activity.

#### 5.5. The Interaction of Beef Heart SMP with (<sup>14</sup>C) DCCD

As a preliminary experiment the binding characteristics (<sup>14</sup>C) - DCCD to beef heart SMP were determined. The results are summarized in Table 5.1. which show that when (<sup>14</sup>C) - DCCD was applied to beef heart SMP at a concentration of 1.0 nmole DCCD/mg SMP protein, and the SMP then washed twice, in buffered sucrose, there was still 0.92 nmole DCCD/mg SMP protein bound to the washed SMP. The ATPase activity of the SMP, before washing, was 57.5% inhibited and the ATP-driven pyridine nucleotide reduction completely inhibited. The (<sup>14</sup>C) - DCCD treated SMP were then extracted with chloroform-methanol, (2:1 v/v) as described by Cattell et al.<sup>70</sup> The chloroform-methanol extract was then washed with  $\frac{1}{5}$ th its volume of distilled water. This washing actually removed very little of the radioactivity, but during the centrifugation

TABLE 5.1.

RECOVERY OF RADIOACTIVITY DURING PREPARATION OF THE  
PROTEOLIPID FROM BEEF HEART SMP LABELLED WITH 1.0 NMOL  
(<sup>14</sup>C) - DCCD/MG PROTEIN

The experiment was performed as  
described by Cattell et al<sup>70</sup>  
and in the text.

Sample	% Yield of ( $^{14}\text{C}$ ) - DCCD
Washed ( $^{14}\text{C}$ ) - DCCD SMP (0.92 nmole DCCD/mg protein)	100
Chloroform-methanol extract of ( $^{14}\text{C}$ ) - DCCD SMP	91.5
Washed chloroform-methanol extract	70.2 *
Ether precipitate from chloroform-methanol extract	34.0
Ether supernatant from ether/chloroform-methanol solution	27.0
Peak eluting off LH-20 column with 2:1 chloroform- methanol	27.5

\* Some material was lost at this stage.

to separate the organic and aqueous phases a centrifuge bottle broke, resulting in loss of material. This is indicated in Table 5.1. The washed organic phase was then concentrated under reduced pressure at 35°C to  $\frac{1}{5}$ th. its volume. After cooling to -20°C this concentrated extract was treated with 5 volumes of diethyl ether, also at -20°C. After keeping the solution at -20°C overnight the resulting precipitate was redissolved in a minimum of chloroform-methanol (2:1, v/v).

This was the crude proteolipid, or DCCD binding protein fraction, which was then applied to a Sephadex LH-20 column equilibrated in chloroform. The column was eluted with various chloroform-methanol solutions, as described,<sup>70</sup> the last being a 2:1 (v/v) chloroform-methanol solution.

It was in this last solution that 81% of the radioactivity originally applied to the column came off as a single peak. This represents 27.5% of the radioactivity that was bound to the beef heart SMP. Cattell et al<sup>70</sup> have shown that the radioactivity in the peak eluting from the LH-20 column is bound to a single protein, which is in a homogenous form, with no other protein contaminants, as seen by polyacrylamide gel electrophoresis. They show that a 32 fold purification in the specific ( $^{14}\text{C}$ ) - DCCD binding has been achieved by this purification process.<sup>70</sup>

#### 5.6. The Interaction of Yeast SMP with ( $^{14}\text{C}$ ) - DCCD

Having shown that ( $^{14}\text{C}$ ) - DCCD bound to beef heart SMP in the manner previously described<sup>10,70</sup> the technique was then applied to yeast SMP, using both D22 WT and D22 A21 strains. ( $^{14}\text{C}$ ) - DCCD was added to the SMP at a concentration causing 60% inhibition of the ATPase activity, respectively 1.0 and 45.0 nmole DCCD/mg. SMP protein for D22 WT and D22 A21 SMP. The SMP were then incubated



for 16 hours at 0°C. However, after two washes with 0.1 M Tris pH 7.5 in 0.25 M sucrose only 3-5% of the applied radioactivity remained associated with the SMP. The yeast SMP ATPase was still inhibited. This observation implied that the mode of DCCD action on yeast SMP might well be different from that on beef heart SMP. However, an alternative explanation was that the binding conditions used for beef heart SMP were not optimal for yeast SMP. Therefore, a series of binding experiments were performed at various pH values, using D22 WT SMP, the results of these experiments being shown in Table 5.2. These show that there is an optimal association of the radioactivity with the SMP at pH 9.5, but even under these conditions only 6.9% of the original radioactivity was associated with the SMP after three washes. At pH 9.5 and 10.5 the DCCD apparently had a greater inhibitory effect on the SMP ATPase activity, but this was probably due to a loss or inactivation of the SMP ATPase during washing at the high pH. This conclusion is supported by the observation that when the D22 WT SMP ATPase activity is titrated with DCCD using a pH 9.5 preincubation, but with no washing after the incubation, then there is no difference from the pH 7.5 preincubation conditions.

There was, however, a possibility that only a fraction of the DCCD applied to the yeast SMP was reacting with the ATPase and that this low level, (< 5% applied DCCD), alone was responsible for the ATPase inhibition.

This possibility was checked by varying the preincubation conditions and determining the resulting ATPase inhibition. These results are presented in Table 5.3 and they show that maximal inhibition, comparable to that achieved overnight at 40°C, may

TABLE 5.2.

(<sup>14</sup>C)- DCCD BINDING TO D22 WT SMP

D22 WT SMP at 37 mg protein/ml were diluted to a final concentration of 10 mg/ml with 0.25 M sucrose, 1 mM succinate, 5 mM MgCl<sub>2</sub> and 50 mM Tris buffer. 0.5 ml of this suspension was then incubated with DCCD, added as a small volume of an ethanolic solution, to the final concentrations shown in the table. The suspensions were kept at 4°C x 16 hours. 10 ml of the dilution medium was then used to wash the SMP which were collected by centrifugation at 120,000 g x 30 minutes. 100 µl samples were taken for estimation of the radioactivity, the samples were counted for 10 minute periods and the cpm corrected to dpm by the channel ratio method. The glycine ethyl ester in the results marked \* was added at a concentration of 400 nmoles/mg SMP protein 60 minutes prior to the addition of DCCD, the preincubation was at 20°C.

pH of Incubation	DCCD applied to SMP ( $\mu$ moles DCCD/ mg protein)	% of Applied dpm				$\mu$ moles DCCD/mg protein		% Inhibition of ATPase in 3rd Pellet
		1st Sp. n	1st Pellet	2nd Pellet	3rd Pellet	2nd Pellet	3rd Pellet	
7.5	1.23	94.0	6.0	2.76	2.4	0.033	0.023	56
7.5	1.47	90.0	4.6	2.89	1.4	0.042	0.0185	69
8.5	2.57	89.5	9.5	6.42	3.4	0.16	0.067	83
9.5	1.43	76.4	19.0	16.3	6.9	0.23	0.096	82
10.5	1.37	89.1	16.2	8.8	5.4	0.12	0.045	84
7.5 *	1.33	89.1	4.26	3.3	1.7	0.047	0.0182	20

\* plus 400  $\mu$ moles GEE/mg SMP protein

TABLE 5.3.

EFFECT OF INCUBATION CONDITIONS ON THE  
DCCD INHIBITION OF S1P ATPase ACTIVITY

D22 WT S1P at 30 mg protein/ml in 0.25 M sucrose, 0.1 M Tris buffer were incubated with DCCD concentrations, as shown. The ATPase measurements were performed at 30°C, pH 9.5 using the NADH oxidation method as described in Chapter 2, section 2.6. The 50% inhibition concentrations of DCCD were obtained from the titration curves drawn from the values given in the table.

Incubation Conditions	% Inhibition of ATPase Activity (nmoles DCCD/mg SMP protein)					50% Inhibition concentration DCCD (nmoles DCCD/mg protein)
	0.1	0.25	0.5	1.0	5.0	
pH 7.5, 4°C x 16 hours	7	15	35	53	80	0.75
pH 7.5, 30°C x 1 hour	14	22	44	54	83	0.75
pH 7.5, 30°C x 1½ hours	19	30	37	55	91	0.8
pH 7.5, 30°C x 2 hours	11	-	27	50	84	1.0
pH 9.5, 37°C x 1 hour	5	-	28	52	86	1.0

be obtained by incubating the D22 WT SMP with DCCD for 1 hour at 30°C.

If the preincubation at 30°C was continued for longer than 1 hour then both the sensitivity of the SMP ATPase to DCCD and the ATPase activity itself declined. No difference was detected in the response of the D22 WT, D22 A16 and D22 A21 SMP ATPase activity to the changes in the pre-incubation conditions.

Since variation in both the temperature and pH of the incubation with DCCD had failed to alter the % inhibition of ATPase activity by a given concentration of DCCD it seemed unlikely that only a small percentage, < 5%, of the applied DCCD was responsible for the inhibition of SMP ATPase activity.

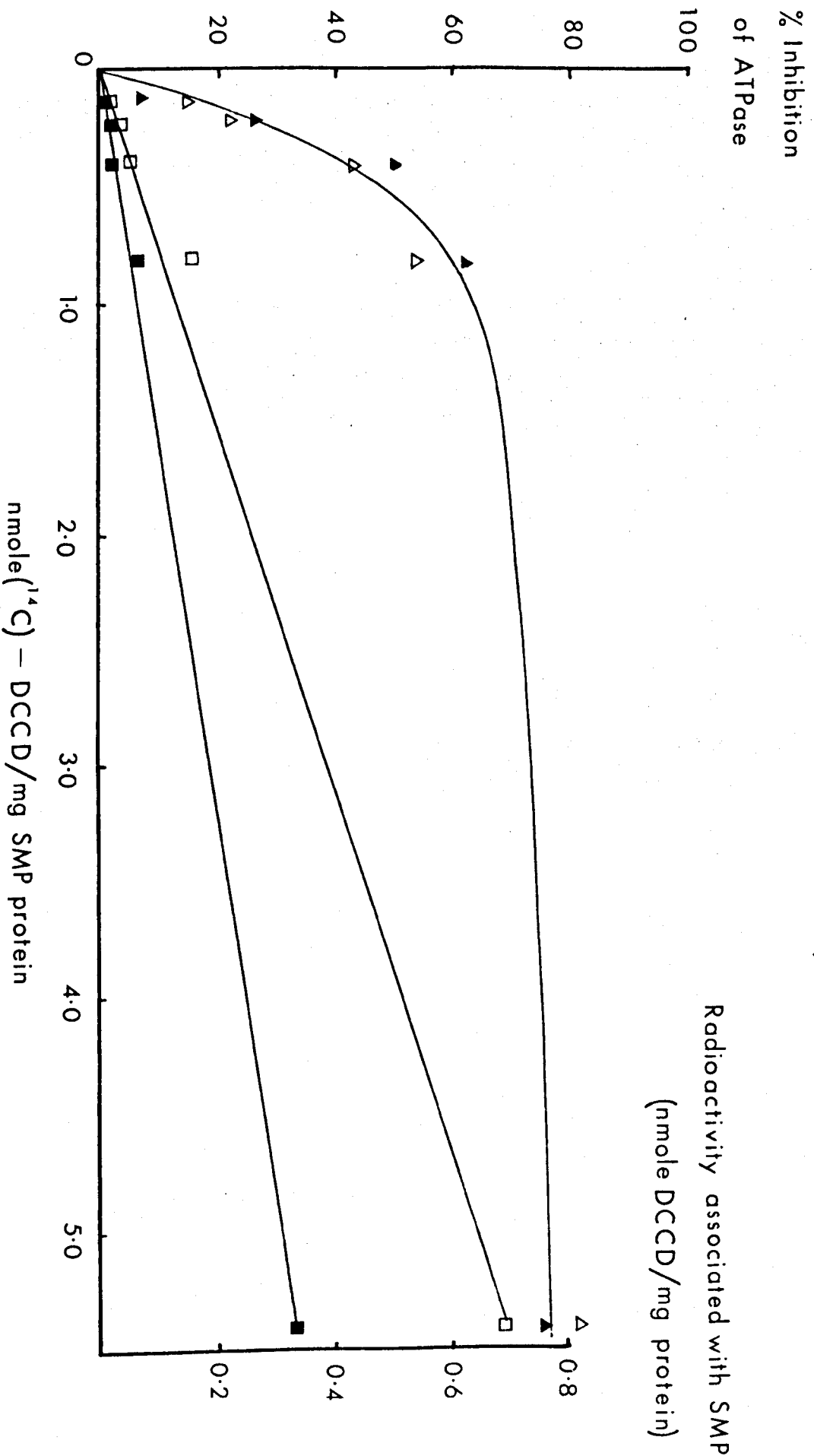
(<sup>14</sup>C) - DCCD was then used to compare the concentration of DCCD associated with the SMP and the inhibition of SMP ATPase activity. The results of this experiment are illustrated in Figure 5.3, which shows that the amount of radioactivity associated with the yeast SMP after washing with 0.25 M sucrose, 0.1 M Tris. pH 7.5 has a linear relationship with the concentration of (<sup>14</sup>C) - DCCD originally applied to the SMP. The amount of radioactivity is shown to decrease with washing the yeast SMP. However, the biological activity, as measured by inhibition of the ATPase, is not a linear relationship with the concentration of (<sup>14</sup>C) - DCCD applied to the SMP. Further, no alteration in the ATPase inhibition was seen after the SMP had been washed a second time, but the concentration of radioactivity associated with the SMP had been decreased approximately 50% by this wash.

From this experiment it was apparent that DCCD was not inhibiting the yeast SMP ATPase activity by irreversibly binding

FIGURE 5.3.

( $^{14}\text{C}$ ) - DCCD BINDING TO D22 WT SMP AND  
INHIBITION OF THE SMP ATPase ACTIVITY

1.0 ml. of D22 WT SMP at 10 mg protein/ml. in 0.25 M sucrose, 0.1 M Tris pH 7.5 were incubated with ( $^{14}\text{C}$ ) - DCCD at the indicated concentrations for 1 hour at 30°C. The % inhibition of the ATPase activity was determined ( $\Delta$ ) and then the SMP were diluted with 15 ml. of the buffered sucrose at 0°C. The SMP were collected by centrifugation at 120,000 g x 30 mins. The radioactivity in this SMP pellet was determined, ( $\square$ ). The washing was repeated and the radioactivity associated with the SMP was also determined ( $\blacksquare$ ). The % inhibition of the ATPase in this pellet was also determined ( $\blacktriangle$ ). ATPase assays were performed at 30°C and pH 9.5 by the NADH oxidation method described in Chapter 2, section 2.10.





to the membrane, as in the case with beef heart SMP. A possible explanation was that the DCCD was acting as a condensing agent, similar to the action of EDAC on the red blood cell ATPase.<sup>79</sup> This hypothesis requires that all the applied carbodi-imide should be converted to the urea form, as was illustrated in Figure 5.1. Thus the nature of the radiolabel after incubation of ( $^{14}\text{C}$ ) - DCCD with yeast SMP was determined. This experiment is illustrated in Figure 5.4, which shows the radioscan of TLC plates run of chloroform-methanol extracts from yeast SMP incubated with ( $^{14}\text{C}$ ) - DCCD and also a control incubation without the yeast SMP.

It can be seen that in the control the radioactivity still is in the form of DCCD, peak Rf 0.95, whilst the majority of the radioactivity in the experimental incubation of SMP + ( $^{14}\text{C}$ ) - DCCD is in a peak Rf 0.35. This is the same mobility as standard ( $^{14}\text{C}$ ) - dicyclohexyl urea and the radioactivity after the incubation was identified as such using mass spectroscopy. In the SMP incubation no radioactivity could be detected at the origin of the TLC plate, which would have indicated a protein bound radioactive complex.

Therefore, it seemed apparent that the behaviour of DCCD with yeast SMP was similar to that of EDAC and red blood cell membranes.<sup>79</sup>

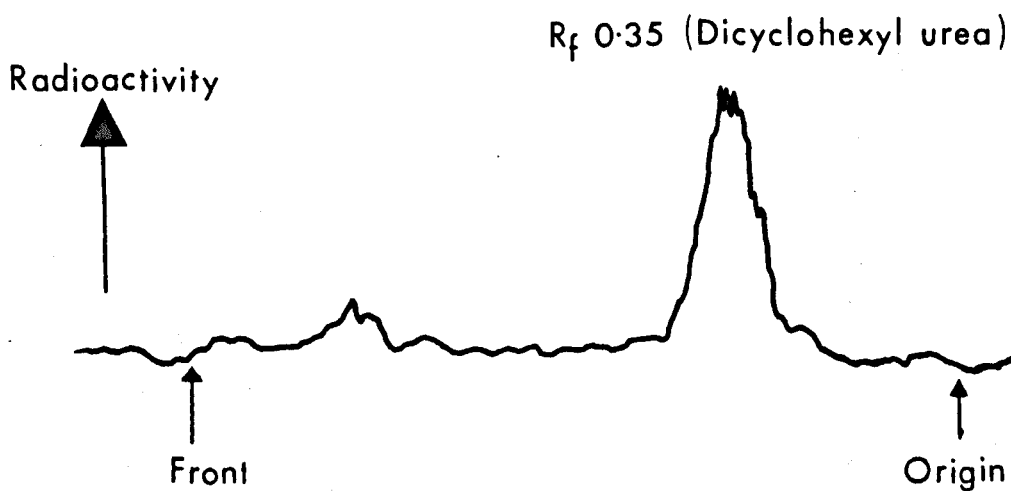
To investigate this possibility more fully the effect of glycine ethyl ester, GEE, on the inhibition of the SMP ATPase activity by DCCD was determined. As had been shown in Table 5.2 if GEE was added to D22 WT SMP at 400 nmoles GEE/mg. SMP protein prior to the addition of 1.3 nmoles DCCD/mg. protein the ATPase activity was only 20% inhibited, compared to the normal 60-65% inhibition observed with this concentration of DCCD.

This protective effect of GEE is further illustrated in

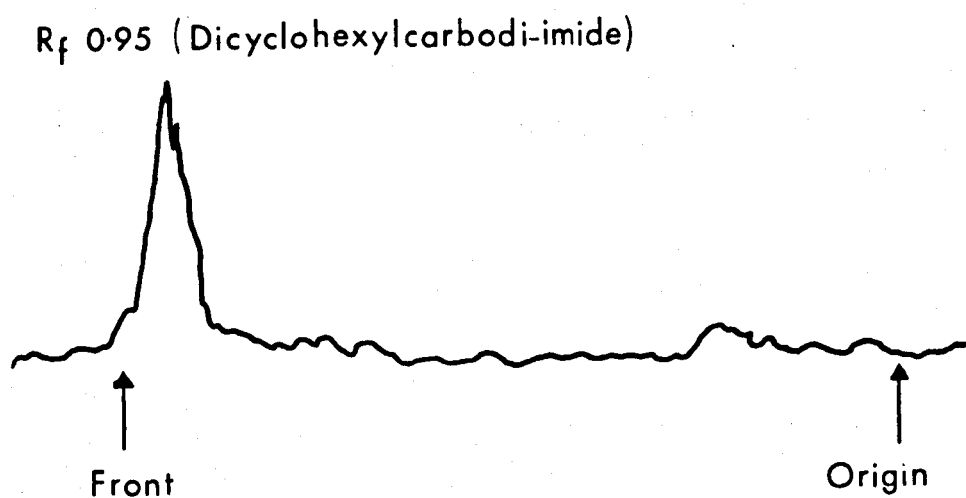
FIGURE 5.4.

NATURE OF THE RADIOACTIVITY AFTER INCUBATION  
OF ( $^{14}\text{C}$ ) - DCCD WITH D22 WT SMP

0.5 ml of D22 WT SMP (40 mg protein/ml) were incubated with ( $^{14}\text{C}$ ) - DCCD at 1 nmole DCCD/mg protein for 1 hour at 30°C. A control incubation was performed with the same amount of ( $^{14}\text{C}$ ) - DCCD in 0.25 M sucrose, 0.1 M Tris pH 7.5. A chloroform-methanol extraction, as described in Chapter 2, section 2.6, was performed without any prior washing of the yeast SMP. The organic phase, after washing with distilled water, was concentrated under reduced pressure at 35°C and then the concentrated extract was run on an alumina TLC plate with benzene-ethyl acetate (9:1, v/v) as described in Chapter 2, section 2.7. The figure shows the radioactivity scans of the TLC plates.



a) Sample, D22 WT SMP + 1 nmole ( $^{14}\text{C}$ )-DCCD / mg SMP protein



b) Control, ( $^{14}\text{C}$ )-DCCD in 0.25 M Sucrose + 0.1 M Tris pH 7.5

Figure 5.5. The inhibition obtained with 1 nmole DCCD/mg SMP protein was titrated against increasing concentrations of ( $^{14}\text{C}$ ) - GEE which were applied prior to the DCCD. Also shown is the DCCD-dependant binding of ( $^{14}\text{C}$ ) - GEE to the yeast SMP. It can be seen that with increasing concentrations of ( $^{14}\text{C}$ ) - GEE there is a progressive decrease in the inhibition caused by the DCCD, and also a corresponding rise in the amount of ( $^{14}\text{C}$ ) - GEE bound to the SMP. Association of ( $^{14}\text{C}$ ) - GEE with the SMP which was not dependant on the presence of DCCD was subtracted from the total amount of radioactivity associated with the SMP, to derive the ( $^{14}\text{C}$ ) - GEE binding dependant on the presence of DCCD.

The concentration of DCCD-dependant binding of ( $^{14}\text{C}$ ) - GEE after the three washes was 0.025 nmoles ( $^{14}\text{C}$ ) - GEE bound/mg SMP protein from an applied concentration of 100 nmoles ( $^{14}\text{C}$ ) - GEE/mg SMP protein. This was in the presence of 1 nmole DCCD/mg SMP protein.

The amount of ( $^{14}\text{C}$ ) - GEE bound to the yeast SMP with increasing concentrations of DCCD was also determined, as shown in Figure 5.6. This ( $^{14}\text{C}$ ) - GEE binding has a linear relationship with the amount of DCCD applied to the yeast SMP, over the DCCD concentration range tested. Also shown in Figure 5.6 are the D22 WT SMP ATPase inhibition curves by DCCD, in the presence of 200 nmoles GEE/mg SMP protein and in the absence of GEE. These curves show that the 50% inhibition values are respectively 2.15 nmoles DCCD/mg protein and 0.75 nmoles DCCD/mg protein with and without 200 nmoles GEE/mg protein.

Polyacrylamide gels were ran of the ( $^{14}\text{C}$ ) - GEE labelled

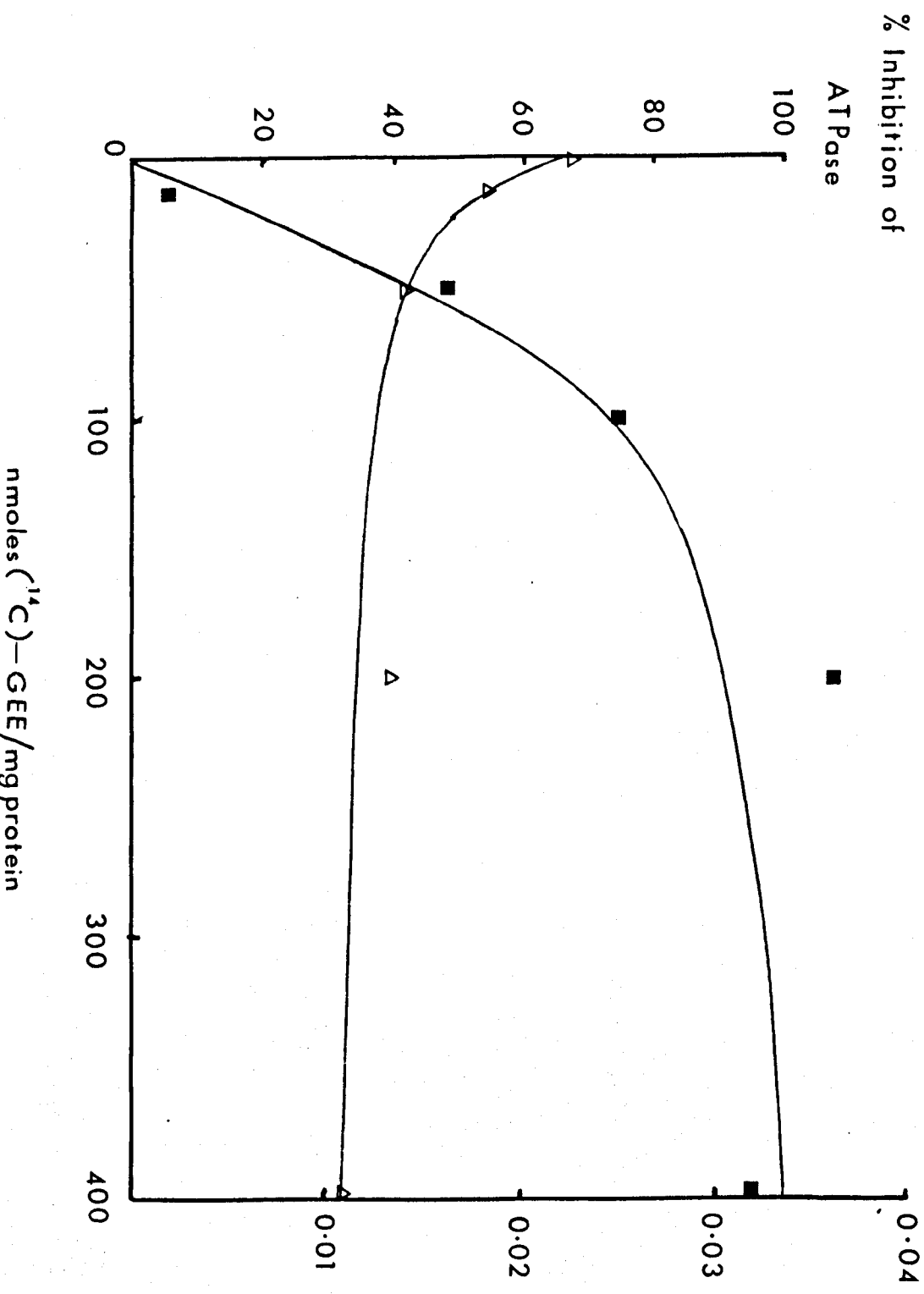
FIGURE 5.5

DCCD - DEPENDANT BINDING OF ( $^{14}\text{C}$ ) - GEE TO

D22 WT SMP.

0.5 ml of 10 mg protein/ml D22 WT SMP were incubated at pH 7.5 with ( $^{14}\text{C}$ )-GEE at the concentrations shown for 60 minutes at 20°C. 1 nmole DCCD/mg SMP protein was then added to each incubation and the SMP were kept at 4°C for 14 hours. The % inhibition of the ATPase activity was determined, ( $\Delta$ ), then the SMP were washed with 15 ml of 0.25 M sucrose, 0.1 M Tris pH 7.5. The SMP were collected by centrifugation at 120,000 g for 30 minutes. This washing procedure, for each incubation, was repeated twice, and then the radioactivity associated with the final SMP pellets was measured. Control experiments with no DCCD were also performed, and by subtracting these values from the experimental values of ( $^{14}\text{C}$ )-GEE binding, the the concentration of DCCD-dependant ( $^{14}\text{C}$ )-GEE binding was calculated, ( $\blacksquare$ ).

Radioactivity associated with SMP  
(nmoles GEE/mg protein)



SMF, but the concentration of bound radioactivity was insufficient to detect any specifically labelled protein bands.

The concentration of DCCD - dependant binding of ( $^{14}\text{C}$ ) - GEE is low, approximately 0.03 nmoles ( $^{14}\text{C}$ ) - GEE/nmole DCCD applied to the D22 WT SMF, with a preincubation of 200 nmoles ( $^{14}\text{C}$ ) - GEE/mg SMF protein.

Figure 5.6 also shows that the decrease in inhibition seen with 200 nmoles ( $^{14}\text{C}$ ) - GEE/mg is not constant throughout the range of DCCD concentrations applied to the SMF. At a DCCD concentration of 0.25 nmole/mg protein the inhibition of ATPase activity was 30% in the absence of GEE, but with 200 nmoles GEE/mg protein preincubation the inhibition decreased to 2%. However, using 5 nmoles DCCD/mg protein, and the same concentration of GEE the corresponding inhibition values were 91% without GEE and 84% with GEE preincubation.

It may be that the hydrophobic DCCD is accessible to sites which the water soluble GEE molecule cannot reach. The use of a lipophilic radiolabelled nucleophile such as aniline, or another organic base might well give higher DCCD - dependant labelling of the mitochondrial membranes. Suitable controls would be necessary to ensure that the concentration of base added was not inhibiting the ATPase activity directly.

Godin and Schrier<sup>79</sup> using 500 nmoles ( $^{14}\text{C}$ ) - GEE/mg protein with red blood cell membranes obtained a binding concentration of 48 nmoles GEE/mg protein, but they were using 4.5 nmoles EDAC/mg protein to promote this binding, so that there is the possibility of other sites binding the ( $^{14}\text{C}$ ) - GEE than that concerned with

# FIGURE 5.6

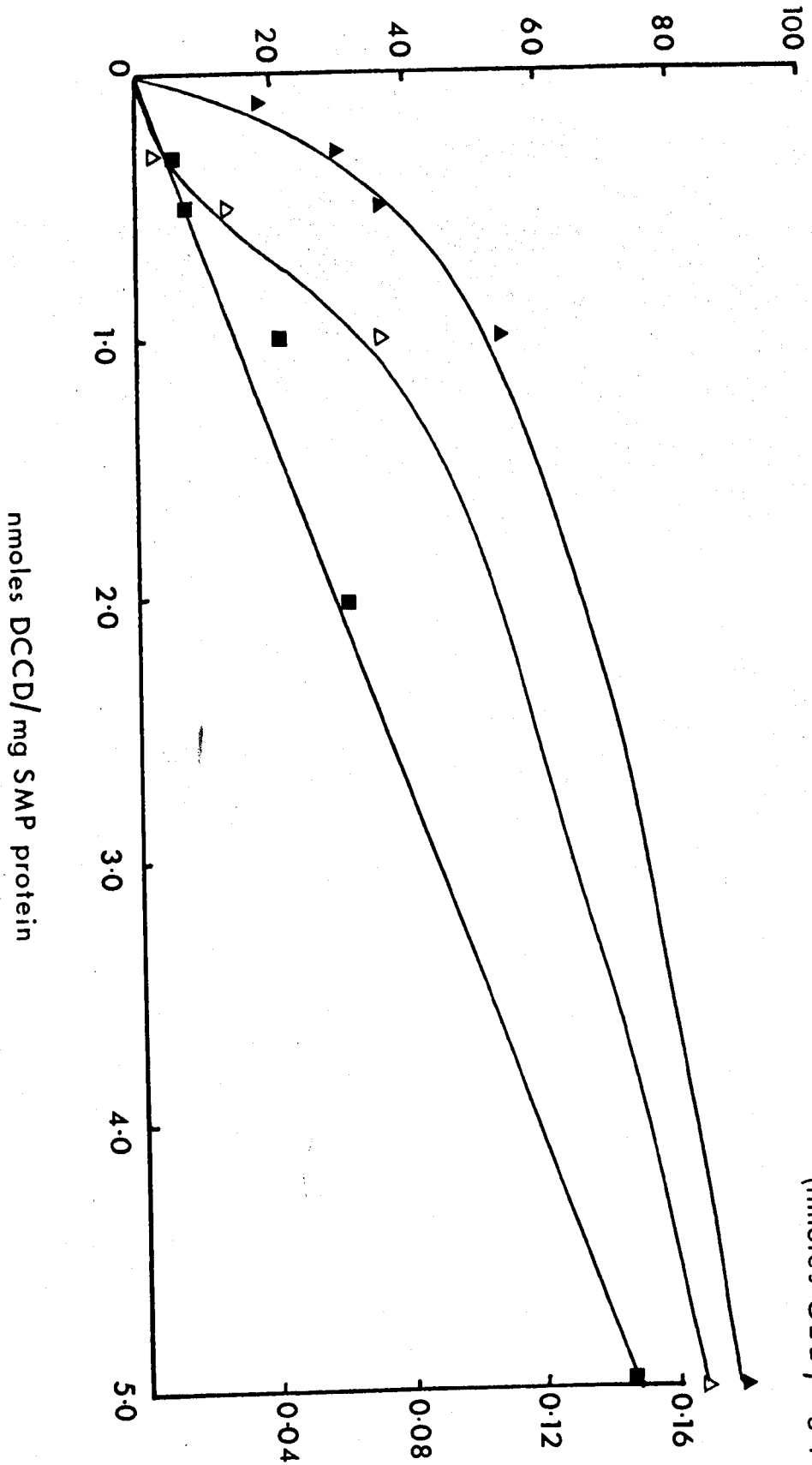
## THE EFFECT OF DCCD ON THE BINDING

OF ( $^{14}\text{C}$ )-GEE TO D22 WT SMP

0.5 ml of D22 WT SMP (10 mg protein/ml) were incubated at pH 7.5 with 200 nmoles ( $^{14}\text{C}$ )-GEE /mg protein for 30 minutes at 30°C. DCCD was then added, at the concentrations shown, and the SMP were incubated for a further 60 minutes at 30°C. The % inhibition of the ATPase activity was then determined, ( $\Delta$ ). 15 ml of 0.25 M sucrose, 0.1 M Tris pH 7.5 was then added to each incubation and the SMP were collected by centrifugation at 120,000 g for 30 minutes. This washing was repeated four times and the radioactivity associated with the final SMP pellet was measured. By a similar series of control experiments to those described in the legend to Figure 5.5 the DCCD-dependant binding of ( $^{14}\text{C}$ )-GEE was calculated, ( $\blacksquare$ ). A DCCD inhibition curve of D22 WT SMP ATPase activity in the absence of GEE is also shown, ( $\blacktriangle$ ).



% Inhibition  
of ATPase



DCCD-dependent  
radioactivity associated with SMP  
(nmoles GEE / mg protein)

ATPase inhibition. Stekhoven<sup>72</sup> has shown that ( $^{14}\text{C}$ ) - DCCD binds to more than one protein when it is applied to beef heart SMP at concentrations greater than that necessary for ATPase inhibition.

An experiment was performed to examine the possibility that a complex was being formed between free GEE and DCCD. 7 nmoles of ( $^{14}\text{C}$ ) - DCCD were incubated with 2.8  $\mu\text{moles}$  GEE in 0.25 M sucrose, 0.1 M. Tris pH 7.5  $4^{\circ}\text{C}$  x 16 hours. A spot of this solution was analysed by TLC using an alumina TLC plate with the benzene:ethyl acetate solvent described in Chapter 2, section 2.7. The plates were scanned for radioactivity, but no difference was seen between the experimental ( $^{14}\text{C}$ ) - DCCD + GEE incubation and a control incubation of ( $^{14}\text{C}$ ) - DCCD alone.

#### 5.7. Discussion and Conclusions

The results presented here confirm the earlier observations of Avner and Peers<sup>80</sup> who found that very low levels of radioactivity were associated with yeast mitochondria after treatment with inhibitory concentrations of ( $^{14}\text{C}$ ) - DCCD. These initial observations may now be extended to the conclusion that the mode of DCCD action differs in beef heart SMP and yeast SMP. In the former case DCCD inhibits by direct interaction with some membrane group, forming a stable covalent bond, whilst with yeast SMP DCCD is primarily acting as a condensing agent, promoting the formation of an intramembrane bond. This postulate for the mechanism of DCCD inhibition of the yeast SMP ATPase is illustrated in Figure 5.7. As explained in the legend to Figure 5.7 the proposed mechanism is the same as that given for EDAC inhibition of the red blood cell ATPase by Godin and Schrier.<sup>79</sup>

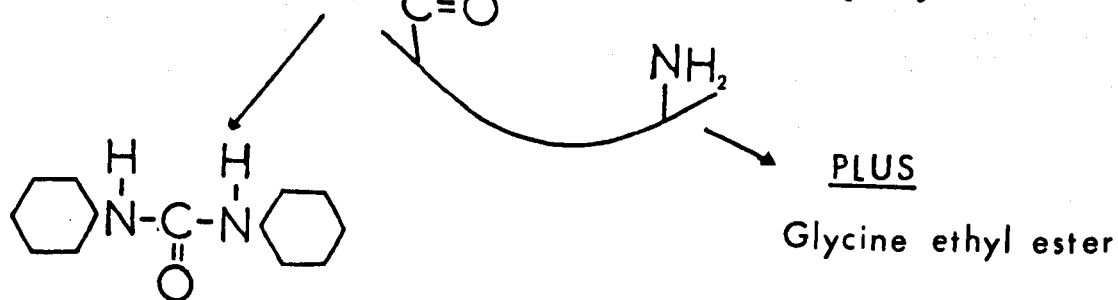
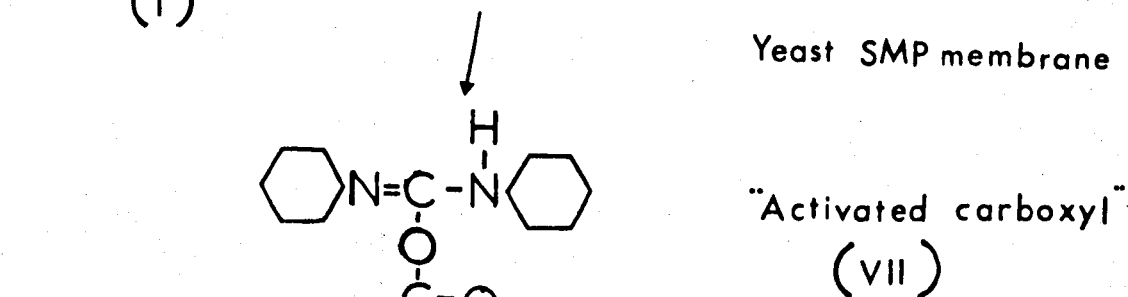
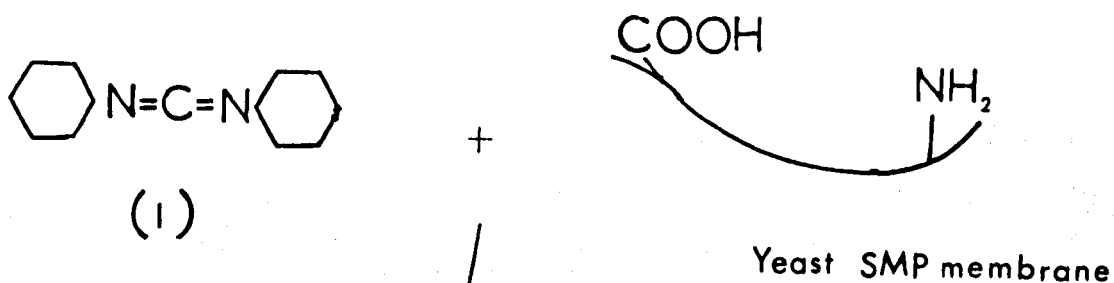
The formation of the intramembrane bond (IX) causes a change in the structure and conformation of the membrane which leads to

FIGURE 5.7.

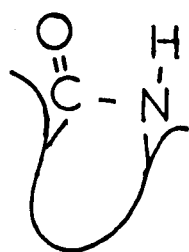
POSTULATED MECHANISM FOR THE INHIBITION OF YEAST SAP  
ATPase ACTIVITY BY DCCD AND THE PROTECTION BY GEE OF  
THIS DCCD INHIBITION

The DCCD reacts with a membrane group, here represented by a carboxyl, to form an activated group, (VII), and in the absence of an added nucleophile, such as GEE, the unstable group (VII) reacts further to yield an intramembrane bond, (IX) and free dicyclohexyl urea (VIII). The intramembrane bond, (IX), is formed by the activated carboxyl and another membrane group, here represented by an amino group.

However, if the GEE is added to the system in sufficient quantity, then this will react with the activated carboxyl (VII), competing with the membrane nucleophile, and thus preventing the formation of the intramembrane bond by forming the complex (X) instead.

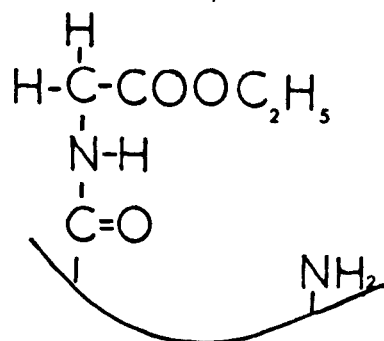
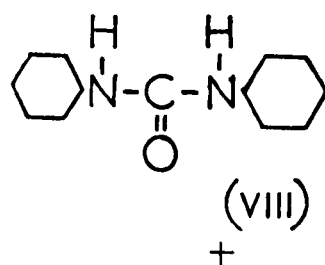


Dicyclohexyl urea (VIII)



Intramembrane bond (IX)

ATPase inhibited



Membrane-bound GEE (X)

ATPase active

the ATPase activity being inhibited. There is an alternative explanation to the DCCD inhibition, if the membrane nucleophile, represented here by an amino group, is an essential group for ATPase activity and, therefore, must remain free. However, this does not seem likely if the proposed mechanism for DCCD inhibition of beef heart mitochondrial ATPase is considered. This is illustrated in Figure 5.8, the essential difference between this mechanism and that for yeast is that the activated carboxyl group, (XI), undergoes a rearrangement to form a stable N-acyl urea bound to the membrane (XII). For both the yeast and beef heart mechanisms to be comparable one must eliminate the membrane nucleophile in the case of beef heart mitochondria, so that the N-acyl urea will be formed and not an intramembrane bond as in the case of yeast. Can therefore this membrane nucleophile be the active moiety in the yeast SMP ATPase whose blockage causes inhibition? In what must be a basically similar enzyme sequence in both yeast beef heart mitochondria it would seem unlikely that in one case the nucleophile was vital to the enzyme function, whilst being absent in the other.

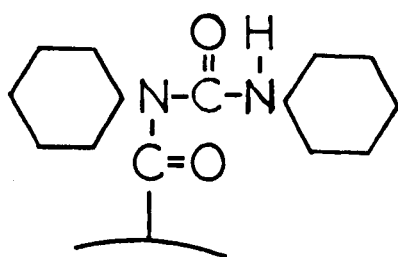
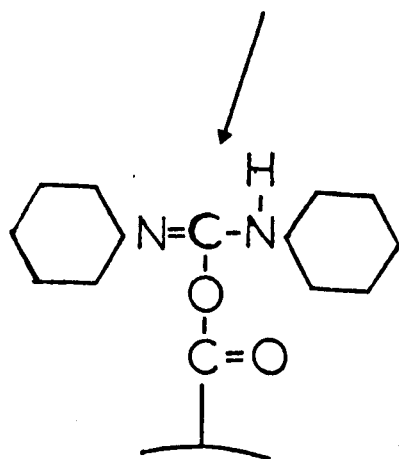
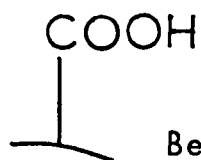
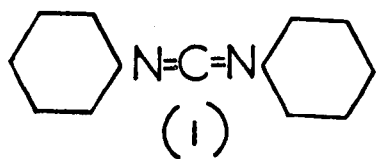
A more reasonable explanation is that in both beef heart and yeast SMP inhibition by DCCD is caused by an alteration in membrane conformation, in the yeast SMP this is caused by an intramembrane bond being formed, whilst in beef heart mitochondria the presence of the N-acyl urea bound to the membrane is sufficient to inhibit enzyme activity.

The observation that after washing the ( $^{14}\text{C}$ ) - DCCD treated yeast SMP there is still a small amount of radioactivity

FIGURE 5.8.

POSTULATED MECHANISM FOR DCCD INHIBITION OF

BEEF HEART SMP ATPase



ATPase inhibited

associated with the membranes and that this association is optimal at pH 9.5 may be significant. In the yeast SMP the derivative (VII) of DCCD action on the carboxyl group has two alternative paths, either to participate in the formation of the intramembrane bond (IX), or possibly to rearrange to form a stable N-acyl urea (XII), as in beef heart mitochondria.

Probably the majority of the activated carboxyl (VII) goes by the former reaction path and free dicyclohexyl urea is a reaction product. However, a small proportion may form the N-acyl urea, thus binding some of the radioactivity to the yeast SMP. At pH 9.5 the membrane conformation may be altered so that the formation of the N-acyl urea may be more favoured under these conditions than at a neutral pH.

This might explain the results presented in Table 5.2, showing that at pH 9.5 6.9% of the original radioactivity applied is still associated with the yeast SMP after three washes, compared to the 1.4 - 2.4% when the ( $^{14}\text{C}$ ) - DCCD and SMP were incubated at pH 7.5. The results for the incubations at all the pH's tested do appear to indicate that there is some association of the radioactivity with the yeast SMP, although this is only a small percentage of the total radioactivity originally present in the incubation.

The site of DCCD action is still uncertain. Evidence has been presented earlier to support the present conclusion that DCCD acts, like oligomycin, by preventing the formation of the high energy phosphorylated intermediate,  $\text{X}\sim\text{P}$ , from the high energy non-phosphorylated intermediate,  $\text{X}\sim\text{I}$ .

How this is achieved is not clear, one possibility is that the DCCD might interact with the protein 'X' directly complexing



the group with which phosphate forms a high energy bond. The high energy non-phosphorylated intermediate appears to be unaffected by DCCD inhibition since mitochondrial energy-linked partial reactions driven by respiratory chain activity are unaffected by DCCD. This implies that  $X \sim I$  is still being formed by the active respiratory chain and that this  $X \sim I$  may be used to drive the partial reactions.

An alternative explanation to the inhibition of  $X \sim P$  formation by DCCD is that instead of acting directly on 'X' by complexing with the active centre at which phosphate would have formed a high energy bond the action of DCCD is merely to prevent access of the phosphate to this site. An obvious mechanism for this would be a local membrane rearrangement under the influence of DCCD, thus denying the inorganic phosphate access to the reaction centre. Alternatively an active group which is necessary to arrange the phosphate in a suitable conformation such that the phosphate can form ' $X \sim P$ ' may be complexed by the action of DCCD on the SMP membrane.

The concentration of GEE binding due to DCCD interaction with the yeast SMP was low, compared to that found by Godin and Schrier<sup>79</sup>, but these workers were using much higher concentrations of EDAC than the concentrations of DCCD used in this study. Also they managed to prevent any inhibition of the ATPase by the EDAC using the high concentrations of GEE. With the yeast SMP used in this study the maximum relief of DCCD inhibition obtained with 400 nmoles GEE/mg protein was a drop from 67% to 33% caused by 1 nmole DCCD/mg protein, as is shown in Figure 5.5. The different

structure and composition of the red blood cell and mitochondrial membranes may explain the low level of ( $^{14}\text{C}$ ) - GEE binding seen with the yeast SMP and a nucleophile more hydrophobic than glycine ethyl ester may well be more accessible to the hydrophobic environment at the site of DCCD action. If a higher concentration of nucleophile binding to yeast SMP could be achieved, this may prove suitable for identifying the protein on which DCCD and the nucleophile are acting, using techniques such as polyacrylamide gel electrophoresis.

The experiments with the DCCD dependant ( $^{14}\text{C}$ ) - GEE labelling of yeast SMP have, however, further indicated the role of DCCD in the inhibition of SMP ATPase activity. The scheme illustrated in Figure 5.7, which accounts for all the experimental findings so far, shows that in the presence of glycine ethyl ester the group with which the DCCD had originally reacted, i.e., the membrane carboxyl in Figure 5.7, is still complexed, (X), but the ATPase activity is not now inhibited. This suggests that the primary reason for DCCD inhibition of SMP ATPase cannot be the complexing of the carboxyl group, but must be a rearrangement of the local membrane environment either by the intramembrane bond in the case of yeast SMP or by the presence of a bound N-acyl urea in the case of beef heart SMP.

Unfortunately the use of ( $^{14}\text{C}$ ) - DCCD to study the oligomycin resistant yeast mutants is impractical, since the low concentration of radioactivity associated with the yeast SMP after washing is insufficient for further characterization of a possible DCCD binding protein. However, further experiments with a more

hydrophobic nucleophile than GMP may well prove useful in a comparative study of the WT and mutant yeast SMP.

## CHAPTER 6

### THE PREPARATION AND PROPERTIES OF A SOLUBLE OLIGOMYCIN SENSITIVE ATPase FROM YEAST SMP

#### Introduction

As shown earlier examination of SMP isolated from the parental and mutant yeast strains had failed to reveal any significant differences, with the exception of an observation on the fatty acid content of the membranes. To investigate the significance of this observation, and also in order to detect any more subtle changes that may have occurred in the mutants it was decided to prepare a soluble oligomycin sensitive ATPase complex, (OS ATPase), that was free of other membrane components not directly concerned with the ATPase complex. As well as being oligomycin sensitive these OS ATPase complexes should also have strain dependant differential oligomycin sensitivities for the purpose of this work.

#### 6.1. Oligomycin Insensitive ATPase Preparations

A soluble oligomycin insensitive ATPase was the first of various membrane factors to be isolated in a pure form. Schatz et al<sup>81</sup> have examined this soluble ATPase, or  $F_1$ , prepared from both beef heart and yeast mitochondria. They demonstrated that there were various distinct differences between  $F_1$  prepared from these two sources. Immunologically the two types were distinct, i.e., antisera prepared against yeast  $F_1$  did not inhibit the enzyme activity of beef heart  $F_1$ . Functional differences were also found, yeast  $F_1$  would only stimulate oxidative phosphorylation in depleted beef heart mitochondria if they were deficient in, but not devoid of,

endogenous ATPase. Schatz et al<sup>81</sup> proposed that in these circumstances stimulation by the yeast  $F_1$  was primarily due to an effect on membrane structure rather than a direct participation in phosphate transfer reactions. It was also shown that when hybrid particles were made from beef heart TU particles and yeast  $F_1$  the ATPase activity of the resulting hybrid had the same oligomycin sensitivity as that previously found for beef heart mitochondria, indicating that the determining factor for oligomycin sensitivity is located on the mitochondrial membrane, rather than on the  $F_1$  ATPase complex.

Soluble oligomycin insensitive ATPase complexes have also been isolated from rat liver SM<sup>82</sup> and from the cytoplasmic reticulum of S.faecalis.<sup>124</sup>

## 6.2. Oligomycin Sensitive ATPase Preparations

Unlike the preparation of an oligomycin insensitive ATPase which normally depends on the mechanical disruption of the membrane by sonication or shaking with glass beads the initial solubilization of an OS ATPase is usually accomplished by treatment with non-ionic detergents or dispersive agents such as deoxycholate or cholate, usually in the presence of high salt concentrations. Once solubilized the mitochondrial proteins are fractionated, methods which have been used, include:  $(NH_4)_2SO_4$  treatment,<sup>83</sup> detergent gradient chromatography,<sup>11</sup> and a glycerol gradient.<sup>84</sup>

Studies on an OS ATPase complex derived from beef heart mitochondria have been reported in a series of papers by MacLennan et al and Tzagoloff et al.<sup>83,85-87</sup> Beef heart electron transport particles were solubilized by deoxycholate in the

the presence of KOH and then the solubilized material was fractionated using  $(\text{NH}_4)_2\text{SO}_4$ . The final OS ATPase was 50% inhibited by 0.5  $\mu\text{g}$  oligomycin/mg protein and on polyacrylamide gel electrophoresis two protein bands were seen in addition to those due to the basic oligomycin insensitive ATPase complex.<sup>83</sup> By treating this OS ATPase with 2 M NaBr the complex lost its oligomycin sensitivity. However, the salt-extracted residue could reconfer oligomycin sensitivity to added  $\text{F}_1$  after the NaBr had been removed.<sup>86</sup> By treating this salt extracted residue with  $\text{NH}_4\text{OH}$  the ability to confer oligomycin sensitivity to free ATPase was lost. In the final paper<sup>87</sup> MacLennan and Tzagoloff use this technique to isolate an 'Oligomycin Sensitivity Conferring Protein', (OSCP), from the  $\text{NH}_4\text{OH}$  extract of the NaBr-extracted OS ATPase. This OSCP was shown to have a MW of 18,000 by gel filtration and also it had some 'coupling factor' activity. The relationship of OSCP to other coupling factors will be discussed later.

Swanljung et al<sup>11</sup> have used a KCl/deoxycholate technique to purify an OS ATPase from yeast SMP. The solubilized extract was purified by detergent gradient chromatography using a Sepharose 6B column. The final OS ATPase preparation was found to be dependant on added phospholipid for maximal ATPase activity. Although the complex was oligomycin sensitive the ATPase activity was very labile and the procedure had produced no increase in ATPase specific activity when the final OS ATPase preparation and the original yeast SMP were compared. Tzagoloff and co-workers, 84,88-92 have used a commercial strain of S.cerevisiae as a model for the study of the mitochondrial ATPase complex, and have

recently published a Triton X-100 solubilization technique to prepare an OS ATPase complex.<sup>84,92</sup> This technique is basically that used in this Chapter to prepare an OS ATPase from S.cerevisiae D22.

A beef heart mitochondrial OS ATPase called  $F_1$ -X has been prepared by Van der Stadt et al.<sup>93</sup> In an elegant series of recombination experiments it was shown that  $F_1$ -X is actually a complex between  $F_1$  and OSCP, which when prepared from beef heart mitochondria do apparently associate in the absence of mitochondrial membranes, unlike  $F_1$  and OSCP derived from yeast.<sup>90</sup> Using SDS polyacrylamide gel electrophoresis  $F_1$ -X was shown to consist of 5 protein bands, 4 corresponding to  $F_1$  and one representing 'X' or OSCP. It should be noted that there is no reason for connecting the 'X' in  $F_1$ -X to the  $X \sim P$  or  $X \sim I$  mentioned in the previous Chapter.

### 6.3. Other Mitochondrial Membrane Factors

Various mitochondrial membrane components have been isolated which show 'coupling factor activity', this activity is demonstrated by the ability of these isolated components to stimulate mitochondrial partial reactions when added to depleted mitochondrial particles which have only a very low native partial reaction activity. One of the earliest coupling factors,  $F_1$ , has ATPase enzyme activity itself, although the other factors isolated do not have any inherent enzyme activity. The concept has been useful in understanding membrane structure and function but it has been complicated by the use of factors which are not necessarily pure preparations.

OSCP,<sup>87</sup> can be prepared from  $F_4$ ,<sup>94</sup> the factor originally reported to be associated with structural protein. The preparation of  $F_2, F_3, F_4, F_6$  and OSCP is a basically similar technique using alkaline solubilization of the mitochondrial membrane. Beechey *et al*<sup>95</sup> have postulated the existence of a 'Basic Coupling Factor' which is a basic protein, eluting from a CM cellulose chromatography column with high ionic strength buffers. Thus some or all of the various non-enzymic factors may be variations of the same protein in different degrees of purification or altered conformational states.

Sanadi and his group of workers have isolated another series of factors, A, B, C, D and A-D. Factor A shows many similarities to  $F_1$ <sup>96</sup> although high ATPase activity remains latent unless heated. Once activated the ATPase activity, like that of  $F_1$ , is cold labile. Although  $F_1$  and Factor A give very similar patterns on phenol-urea-acetic acid polyacrylamide gel electrophoresis and are also indistinguishable by immunological methods,<sup>97</sup> there are differences between the two, Factor A has 1 mole of tightly bound ADP per mole of Factor A<sup>98</sup> which is not present in preparations of  $F_1$ .

Factor B, MW 29,000 stimulates energy linked reactions in ammonium-EDTA particles and also it can be isolated from preparations of  $F_2, F_3, F_5$ , and  $F_6$ .<sup>98,99</sup> Factor B may also have a close relationship to OSCP since a recent report gave a revised estimate of 29,000 for the MW of OSCP.<sup>100</sup> Both Factor B and OSCP have a tendency to polymerize in low ionic strength solutions. Factor C was isolated by its ability to stimulate the ATP driven reduction of pyridine nucleotide by succinate in highly depleted particles,<sup>101</sup>



this stimulation being greater than that produced by Factor B.

Factor D<sup>97</sup> is obtained as a complex with Factor A during the preparation of the latter, and is distinguished by the degree of stimulation of partial reactions seen with ammonium-MOTA particles.

The complex A.D has an ATPase specific activity of 2-6  $\mu$ moles/min/mg which may be increased to over 90 by heating A.D at 64°C for 2 minutes.<sup>98</sup> Factor A.D also had ATP-Pi and ATP-ADP exchange activities, the former exchange being sensitive to both oligomycin and uncouplers such as TTFB. Fisher et al<sup>102</sup> suggest that these results indicate that a high energy non-phosphorylated intermediate must be formed by the complex and have now called Factor A.D, the 'Soluble Mitochondrial ATP Synthetase Complex'.

Stekhoven<sup>71</sup> has compared Factor A.D and an CS ATPase isolated from beef heart mitochondria. Using SDS polyacrylamide gel electrophoresis techniques he demonstrated a distinct similarity between the two preparations, Factor A.D, having two extra proteins but lacking one protein present in the CS ATPase. Stekhoven reported a total of 16 proteins in Factor A.D, using SDS polyacrylamide gel electrophoresis, which is considerably more than that shown originally by Sanadi et al.<sup>98</sup>

## Results

### 6.4. Preparation of an Oligomycin Sensitive ATPase

Initially the procedure described by Tragoloff and Keagher<sup>84</sup> was used to prepare an CS ATPase complex from S.cerevisiae D22 WT. The technique involved a partial solubilization of the SPT membrane with 0.25% (v/v) Triton X-100 and then the purification of this

FIGURE 6.1.

PREPARATION OF AN OS ATPase FROM YEAST SMP

(Modified from Tzagoloff and Meagher<sup>84</sup>)

All operations at 0 - 4°C

3 ml. SMP (30 - 40 mg. protein/ml.) in ( 0.25 M sucrose  
( 0.1 M Tris, pH 7.5.

Diluted to final protein concentration 6 mg/ml. with 4 mM Tris, pH 7.5. 10% Triton X-100 added to a final concentration of 0.05%, (v/v).

140,000 g x 30 mins.

Pellet

Resuspend to original volume in 4 mM Tris, pH 7.5. 10% Triton X-100 added to final concentration 0.2% (v/v).

Supernatant

10-15% SMP protein, no ATPase activity.

140,000 g x 30 mins.

Pellet  
Discard

Supernatant, 50-60%  
total ATPase activity

10 ml. of supernatant  
layered on to 50 ml. of a  
5-15%, (v/v) glycerol  
gradient containing 4  
mM Tris, pH 7.5 and 0.06%  
(v/v) Triton X-100

53,000 g x 16 hours

ATPase peak removed from  
the gradient concentrated  
by ultrafiltration through  
a  $10^5$  M.W. cut off membrane.  
Final protein concentration  
approximately 1 mg/ml.

extract by centrifugation on a 5-15% glycerol gradient. However, using this procedure it was found that the ATPase activity removed from the glycerol gradient was oligomycin insensitive and also the specific activity of this ATPase was much lower than that reported by Tzagoloff and Meagher.<sup>84</sup> This implied that the ATPase complex was being partially denatured by the procedure, so various modifications were made to the published method.<sup>84</sup> These modifications are listed below:

- (1) A preliminary wash of the SMP with 0.05% (v/v) Triton X-100 before the extraction.
- (2) The Triton X-100 concentration used for extraction was lowered from 0.25% to 0.2% (v/v).
- (3) The Triton X-100 concentration in the glycerol gradient was 0.06% (v/v) and not 0.1% (v/v).
- (4) The final ultrafiltration step used to concentrate the OS ATPase after removal of the ATPase peak from the glycerol gradient was performed using a 100,000 M.W. cut off membrane.

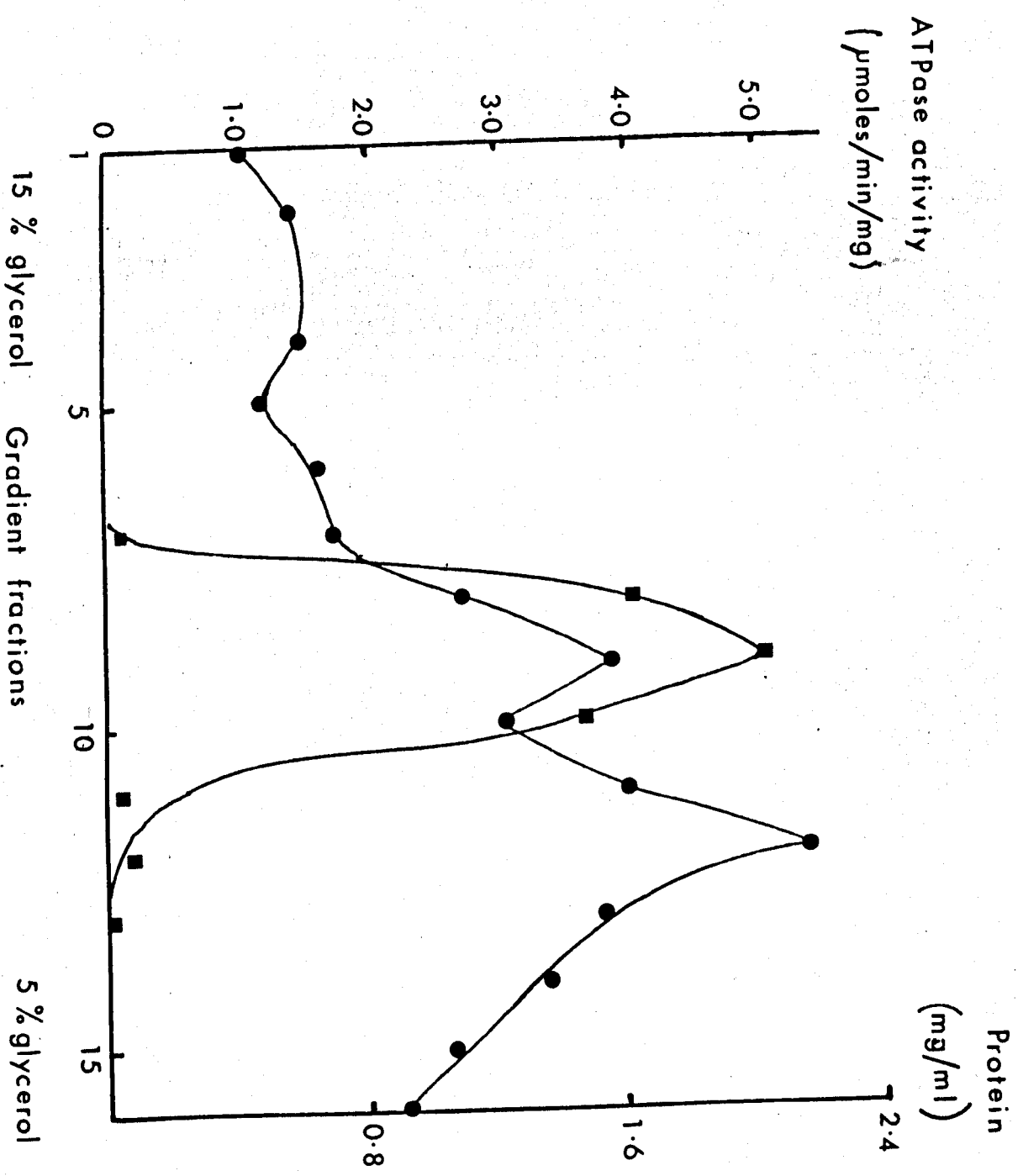
The final procedure used is shown in Figure 6.1. The preliminary wash of the yeast SMP removed 10-15% of the total SMP protein, but no ATPase activity. The amount of ATPase activity removed from the SMP by the 0.2% Triton X-100 did not vary significantly in the strains used, normally 50-60% of the total SMP ATPase activity was solubilized by this treatment.

A typical analysis of the glycerol gradient is shown in Figure 6.2, which is a diagram of a gradient containing a sample of D22 A21 OS ATPase. Protein concentrations and ATPase activity within the gradient are shown. The behaviour of the D22 A16 OS ATPase was the same as that shown for the D22 A21 preparation, the ATPase activity reaching equilibrium slightly above the mid point of the glycerol gradient.

FIGURE 6.2.

ANALYSIS OF THE 5-15% GLYCEROL GRADIENT  
CONTAINING D22 A21 OS ATPase

The gradient was fractionated using an MSE tube piercer coupled to an LKB fraction collector. 4 ml fractions were collected and both the ATPase activity (■), measured at 30°C and pH 9.5, and the protein content (●) in each fraction were determined.



The ATPase complex derived from D22 WT behaved differently from the two mutants described above, since the peak of ATPase activity was normally found at a slightly higher position in the gradient than that of the mutant complexes.

Only one peak of ATPase activity was found on gradients of the D22 WT, D22 A12, D22 A16 or D22 A21 OS ATPase preparations. Also there was no qualitative change in the distribution of protein within the gradient between any of the strains examined.

#### 6.5. Properties of the OS ATPase Complexes

##### (a) Oligomycin Sensitivity.

Even using the modified method it was found that the final D22 WT OS ATPase complex after concentration was oligomycin insensitive. However, both the D22 A16 and D22 A21 ATPase complexes retained oligomycin sensitivity in the final concentrate. Oligomycin sensitivity was defined by testing the effect of 200  $\mu$ g oligomycin/mg protein on the ATPase activity of the complex. If this had no effect then the preparation was termed oligomycin insensitive.

The ATPase activity in the 0.2% Triton X-100 extract was examined for oligomycin sensitivity. The results for D22 WT, D22 A12, D22 A16 and D22 A21, are given in Figure 6.3, which shows the oligomycin inhibition curves of the ATPase activity in this 0.2% Triton X-100 extract 60 minutes after the SMP were homogenized with 0.2% Triton X-100. Also shown is the inhibition curve for the D22 WT ATPase activity in the 0.2% Triton X-100 270 minutes after the membranes had been homogenized in the 0.2% Triton X-100.

It can be seen that the oligomycin sensitivity of the D22 WT ATPase complex has declined from a 50% inhibition concentration of

### FIGURE 6.3.

#### OLIGOMYCIN INHIBITION CURVES FOR THE ATPase ACTIVITY PRESENT IN THE 0.2% TRITON X-100

The ATPase activity was measured at 30°C and pH 9.5 using the  $\text{NADH}_2^+$  oxidation described in Chapter 2. The oligomycin inhibition was measured 60 minutes after the SMP had been homogenized in 0.2% Triton X-100 and also again at 270 minutes for the D22 WT sample.

□	D22 WT, 60 minutes after homogenization				
△	D22 A12, " " " "				
●	D22 A16, " " " "				
○	D22 A21, " " " "				
■	D22 WT, 270 " " "				

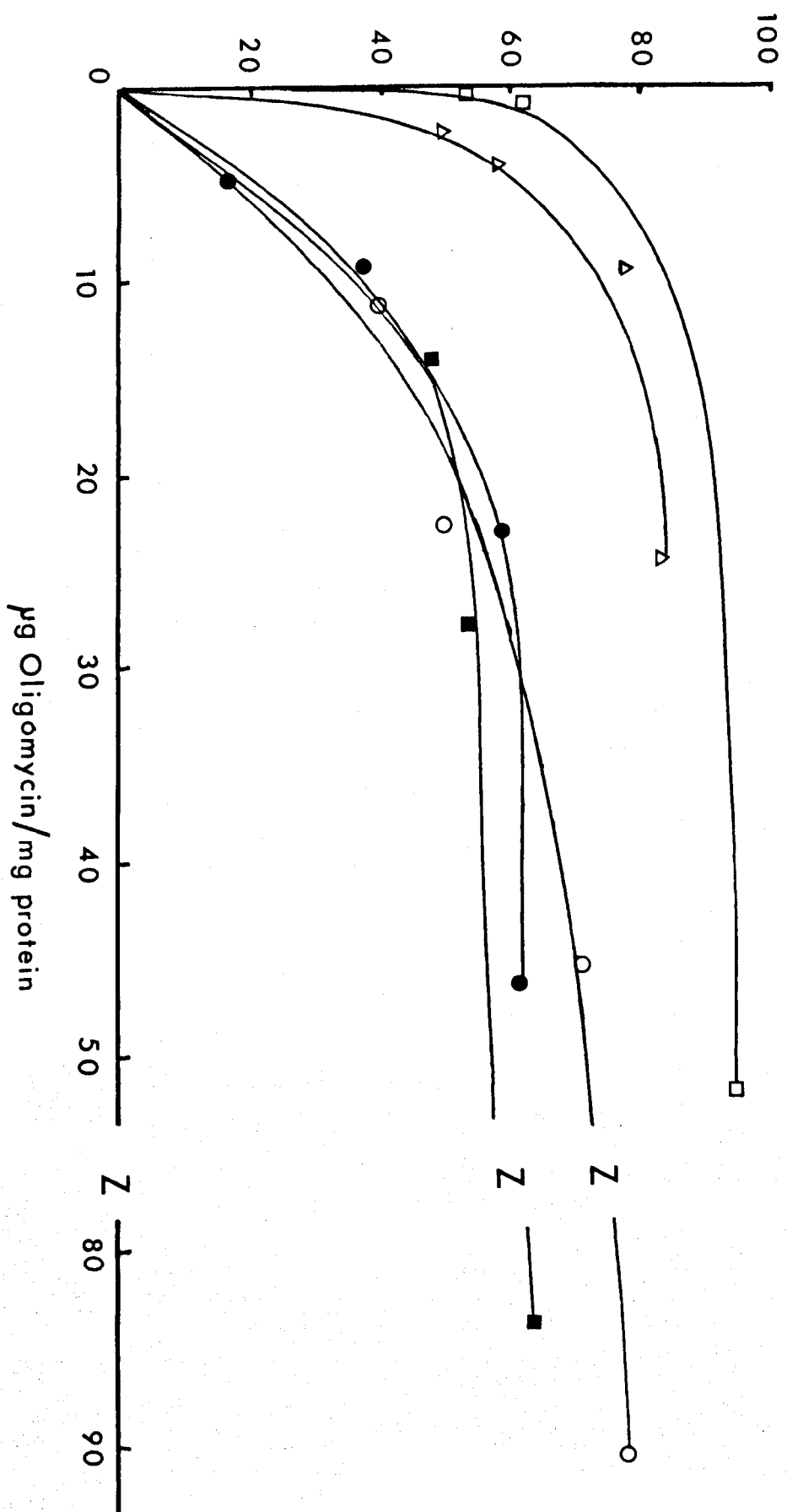
No preincubation period was used in measuring the oligomycin inhibition.

The ATPase specific activities were:

D22 WT, at 60 minutes	- 0.79	μmoles/min/mg			
D22 A12, " " " "	- 2.4	" " "			
D22 A16, " " " "	- 1.53	" " "			
D22 A21, " " " "	- 0.69	" " "			
D22 WT, " 270 " "	- 0.7	" " "			



% Inhibition  
of ATPase



0.27  $\mu\text{g}$  oligomycin/mg protein at 60 minutes to a value of 18.0  $\mu\text{g}$ /oligomycin/mg protein at 270 minutes.

This is further illustrated in Figure 6.4 which shows how the oligomycin inhibition of the ATPase activity in the 0.2% X-100 extract is affected by keeping this extract at 0°C. Fixed concentrations of oligomycin were used for each strain shown, the concentrations being those which were found to give 50-60% inhibition of the ATPase activity in the 0.2% Triton X-100 extract 60 minutes after the membranes had been homogenized in this 0.2% Triton X-100. The effect of 0.27  $\mu\text{g}$  oligomycin/mg protein on the D22 WT extract rapidly declined, so that at 270 minutes this concentration of oligomycin did not have an appreciable effect on the ATPase activity. By contrast the soluble ATPase preparations from D22 A12, D22 A16 and D22 A21 all have a stable oligomycin sensitivity during this period.

270 minutes after homogenization in 0.2% Triton X-100 the soluble ATPase from D22 WT is less sensitive to oligomycin than the membrane bound SFP ATPase, the 50% inhibition concentration of oligomycin was 18  $\mu\text{g}$  oligomycin/mg protein. However, at 60 minutes after homogenization in the 0.2% Triton X-100 the soluble ATPase was apparently more sensitive, the 50% inhibition concentration was 0.27  $\mu\text{g}$  oligomycin/mg protein, compared to the SFP 50% inhibition concentration of 1.5  $\mu\text{g}$  oligomycin/mg protein. The implications of these results will be discussed at the end of the Chapter. D22 A12 shows a similar, though not quite so pronounced increase in sensitivity at this stage. However, the D22 A12 ATPase activity in the 0.2% Triton X-100 extract does not lose oligomycin sensitivity, as has been shown for the

FIGURE 6.4.

STABILITY OF THE OLIGOMYCIN SENSITIVITY OF THE  
ATPase ACTIVITY IN THE 0.2% TRITON X-100 EXTRACTS

The ATPase activity was measured at 30°C and pH

9.5. Oligomycin concentrations used to inhibit  
the ATPase activity were:

□	D22 WT	0.27	μg	oligomycin/mg	protein
Δ	D22 A12	1.9	"	"	"
●	D22 A16	22.6	"	"	"
○	D22 A21	22.0	"	"	"

The Triton X-100 extracts were kept at 0°C during  
the course of the experiment. No preincubation  
was used in determining the oligomycin  
sensitivity of the ATPase preparation.

% Inhibition of

ATPase

100

80

60

40

20

0

60

90

120

150

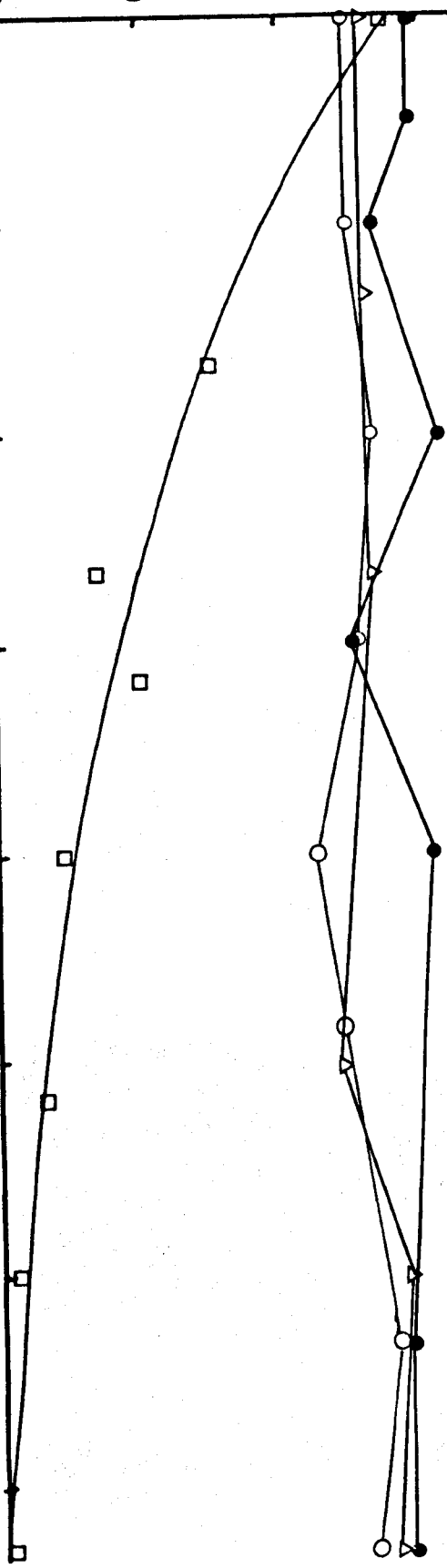
180

210

240

270

Time (mins.) after SMP homogenized in 0.2% Triton X-100



D22 WT preparation. The preparations from strains D22 A16 and D22 A21 behave similarly, retaining the same degree of oligomycin sensitivity in the soluble ATPase complex as in the membrane bound form of the ATPase.

As shown in Table 6.1 with the mutant preparations there is an apparent loss of oligomycin sensitivity after the OS ATPase preparations had been concentrated by ultrafiltration.

This phenomenon was also observed by Tzagoloff and Neagher<sup>84</sup> who gave evidence to support the idea that this is due to a concentration of the Triton X-100 at this stage and that this decreases the oligomycin sensitivity of the ATPase complex by the Triton X-100 forming hydrophobic detergent micelles which competitively accumulate added oligomycin.

#### (b) ATPase activity.

The ATPase specific activities of the various stages in the preparation of the OS ATPase complexes from D22 WT, D22 A12, D22 A16 and D22 A21 are shown in Table 6.2. It can be seen that with D22 A16 and D22 A21 there is a gradual increase in the ATPase specific activity, resulting in a 6 fold increase in the final OS ATPase compared to the original SMP enzyme activity. With D22 A12 there is a 2.5 fold increase in the ATPase activity, but the D22 WT preparation shows no such increase, in fact the specific activity of the final OS ATPase preparation is less than that of the original SMP ATPase. This failure to increase the D22 WT ATPase activity was seen in all the OS ATPase preparations from the D22 WT strains, no matter whether the original D22 WT SMP exhibited a 'high' or 'low' oligomycin sensitivity as described in Chapter 3. Thus the instability of the oligomycin sensitivity observed in the D22 WT

TABLE 6.1.

PREPARATION OF CS ATPase COMPLEX, OLIGOMYCIN

SENSITIVITY OF THE ATPase ACTIVITY

The 50% inhibition values were obtained from full titration curves of the ATPase oligomycin sensitivity. The ATPase activity was measured at 30°C and pH 9.5 using the  $\text{NADH}_2^+$  method described in Chapter 2, section 2.10.

	Concentration of oligomycin giving 50% inhibition of ATPase activity. ( $\mu\text{g}$ oligomycin/mg protein)			
	D22 WT	D22 A12	D22 A16	D22 A21
SMP	1.5	7.0	22.0	22.0
0.2% Triton X-100 extract at 60 mins.	0.27	2.0	16.5	19.0
0.2% Triton X-100 extract at 270 mins.	18.0	No Change	No Change	No Change
0.2% Triton X-100 extract at 24 hours.	No effect at 200 $\mu\text{g}/\text{mg}$	Not tested	Not tested	No Change
ATPase fraction off glycerol gradient after concentration by ultrafiltration through at $10^5$ M.W. cut off membrane	-	200	200	143

TABLE 6.2.

PREPARATION OF OS ATPase COMPLEX, ATPase  
SPECIFIC ACTIVITIES

The ATPase activity was measured at 30°C and  
pH 9.5, using the NADH<sub>2</sub><sup>+</sup> oxidation method  
described in Chapter 2, section 2.10.



	ATPase ( $\mu$ moles/min/mg. protein).			
	D22 WT	D22 A12	D22 A16	D22 A21
SMP membrane	0.72	1.75	0.92	0.57
0.2% Triton X-100 extract at 60 mins.	0.79	2.4	1.53	0.69
0.2% Triton X-100 extract at 270 mins.	0.70	2.0	1.73	0.74
ATPase peak from glycerol gradient.	0.63	4.2	6.3	3.25

OS ATPase preparation was also reflected in the instability of ATPase activity.

The 12.5 fold increase in ATPase specific activity reported by Tzagoloff and Meagher<sup>84</sup> was not observed in any of the S.cerevisiae D22 strains that were examined, the 6 fold increase observed with D22 A16 and D22 A21 being the maximum found. Therefore, there may be some degree of inherent instability in the S.cerevisiae D22 ATPase complexes when they are freed from the SPT membrane and this instability is maximal in the D22 WT strain.

This conclusion is supported by comparing the protein yields of the final OS ATPase preparation reported by Tzagoloff and Meagher<sup>84</sup> with those obtained from S.cerevisiae D22.

These yields were approximately the same i.e., 2 mg OS ATPase protein per 100 mg SPT protein, suggesting that the lower specific activities reported here are not due to impurities present in the OS ATPase, but rather a loss of activity due to a gradual denaturation of the ATPase complex. However, these observations on the strain dependant differential oligomycin sensitivities make the OS ATPase a suitable preparation for a study to determine any detailed differences between the D22 WT and mutant strains which may be connected with the oligomycin resistance observed in the mutants.

#### 6.6. Comparative Analysis of the Components Present in the OS ATPase Complexes

The samples used for comparison between the various strains were the final OS ATPase complexes obtained after the ultrafiltration concentration step.

##### (a) Protein Composition of the ATPase Complex.

The protein components present in the OS ATPase complexes

were analysed by SDS polyacrylamide gel electrophoresis, as described earlier in Chapter 2, section 2.0, using a system modified from that originally given by Weber and Osborn.<sup>19</sup> The gels are shown in Figure 6.5 and it can be seen that there is no alteration in any of the major protein bands in the D22 WT CS ATPase complex compared to that from either D22 A12, D22 A16 or D22 A21. The relative proportions of these proteins were revealed by scanning the gels in a Joyce-Loebel gel scanner and the resultant traces showed that there was no difference in the relative proportions of the proteins from the various CS ATPase complexes.

There is a minor band apparent in all the gels which was not shown by Tzagoloff and Mcagher.<sup>84</sup> This band is labelled 'X' in Figure 6.5 and is just below the heavily stained bands 1 and 2. It is not clear in Figure 6.5 that bands 1 and 2 are separate, but in other gels of the CS ATPase complexes, with less protein being applied, the bands did become clearly defined. Tzagoloff and Mcagher<sup>84</sup> also show that 6 and (7) are another distinct pair, but this was not seen in the gels run of the S.cerevisiae D22 CS ATPase complexes. In these gels bands 6 and (7) are represented as a wide single band, so there may be two proteins present, with very similar molecular weights. When the CS ATPase complexes were run in the other SDS polyacrylamide gel system used to separate the yeast SIF protein components more protein bands were apparent, band 'X' being replaced by three distinct minor bands under these conditions of electrophoresis. Also bands 6 and (7) were seen as a distinct pair, rather than a diffuse single band. However, using this electrophoresis system no strain specific differences in the protein components of the CS ATPase complexes were detected.

FIGURE 6.5.

POLYACRYLAMIDE GELS OF OS ATPase COMPLEXES

The gels shown are of OS ATPase complexes in the modified Weber and Osborn system<sup>19</sup> described in Chapter 2, section 2.9. The gels show, left to right, the OS ATPase complexes from: D22 WT, D22 A12, D22 A16 and D22 A21.

⊖

- 1,2

- x

- 3

- 4

- 5

- 6(7)

- 8

- front

⊕

W 1

A 12

A 16

A 21

(b) Phospholipid Analysis of the OS ATPase complexes.

Chloroform-methanol extractions, as described in Chapter 2, section 2.6, were performed on the OS ATPase complexes and the extracts were analysed by TLC on 0.25 mm silica plates using a chloroform : methanol : ammonia system, (see Chapter 2, section 2.7.).

Qualitatively there was no difference in the extracts of the OS ATPase complexes from D22 WT, D22 A16 and D22 A21 strains, compounds running with the same mobilities as phosphatidyl ethanolamine and cardiolipin being found in all three extracts.

Preliminary quantitative results suggest that more phospholipid is associated with the D22 WT OS ATPase complex than with the OS ATPase complexes from either D22 A16 or D22 A21. Assays were performed to determine the total phosphate associated with the OS ATPase complex. Values of 3.3, 1.15 and 0.75  $\mu$ moles Pi/mg. protein were obtained for the OS ATPase complexes from D22 WT, D22 A16 and D22 A21. Since these assays have not been duplicated with other cultures of the yeast strains the higher value for the D22 WT OS ATPase complex must be regarded as a preliminary and not a definitive result. However, the results presented in the next Chapter support the possibility that there may be more lipid associated with the D22 WT OS ATPase than with the mutant OS ATPase complexes.

(c) Neutral Lipid Analysis of the OS ATPase complexes.

Using the same chloroform-methanol extracts the neutral lipids were examined by TLC on 0.25 mm silica plates using the pet. ether-diethyl ether-acetic acid system described earlier. A variety of components from the chloroform-methanol extracts were

separated by this system, including fractions running with the same Rf values as: hydrocarbons (0.98), esterified sterols (0.92), free fatty acids (0.22), free sterols (0.15), plus other minor fractions running with Rf values less than 0.05.

Using this method no qualitative differences could be found in the chloroform-methanol extracts from OS ATPase complexes of D22 WT, D22 A16 and D22 A21, no individual fraction being exclusively found in any one strain.

Quantitative analysis of the ergosterol fraction from the OS ATPase complexes will be presented in the next Chapter.

#### 6.7. Discussion and Conclusions

Using Triton X-100 as a solubilizing agent a partially purified, soluble oligomycin sensitive ATPase complex has been prepared from yeast SMP. Initially these complexes retained the same relative oligomycin sensitivities that had been found in the yeast SMP. However, there appears to be a strain-specific instability in the ATPase complex when it is solubilized from the SMP membrane. This instability is especially marked in the D22 WT preparation which not only loses ATPase activity after removal from the membrane but also loses the oligomycin sensitivity of the soluble ATPase complex. The mutants D22 A12, D22 A16 and D22 A21 all retain oligomycin sensitivity during the preparation of the soluble ATPase complex. It is suggested, however, that even in these relatively stable mutant preparations there is some loss of ATPase activity compared to the results of the S.cerevisiae strain used by Tzagoloff and Meagher.<sup>84</sup>

No definite conclusions can be reached as to whether the

ATPase complex from the D22 WT strain undergoes a decay into sub units or merely a rearrangement of the enzyme complex in response to the new environment when it is removed from the SMP membrane by the Triton X-100 solubilization. The latter suggestion is supported by observations on the final OS ATPase complex, which shows there was no difference in the protein composition of the oligomycin insensitive D22 WT ATPase complex compared to the oligomycin sensitive complexes derived from D22 A12, D22 A16 and D22 A21. If the D22 WT ATPase complex had degraded into sub units during the preparation one would have expected to find some alteration in the protein composition.

The observation that D22 WT ATPase complex reached an equilibrium position in the glycerol gradient that was higher than found for the mutant OS ATPases suggests that the specific gravity of this complex is less than that of the mutant complexes, which may be accounted for by a change in the lipid composition of the ATPase. Preliminary results do indicate that there is more phospholipid associated with the D22 WT ATPase than the mutant OS ATPase complexes. If this is correct then the D22 WT complex may undergo a greater internal rearrangement than the mutant complexes in order to reach a stable conformation in the aqueous environment, explaining the lower stability of both the ATPase activity and its oligomycin sensitivity observed when the D22 WT ATPase complex is solubilized.

The main conclusion from this work is that the OS ATPase preparation offers a simpler system for investigating the mode of oligomycin resistance found in the yeast mutants. The OS ATPase preparation is less complex in both its lipid and protein composition than the SMP membrane from which it was prepared. These OS ATPase



preparations also retain the differential oligomycin resistance observed in the SMP ATPase activity.

## CHAPTER 7

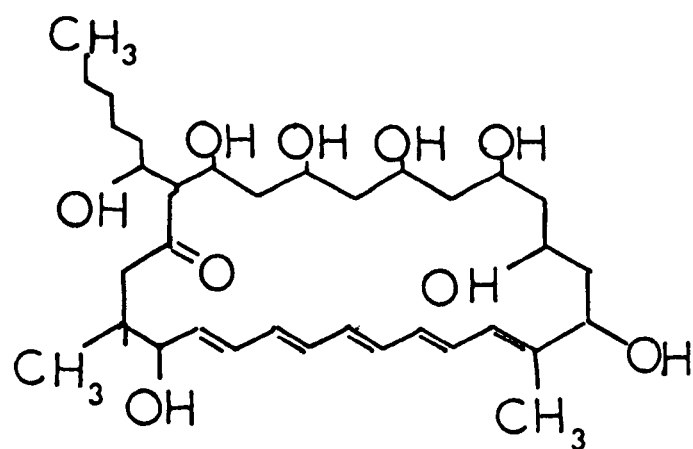
### THE ROLE OF STEROLS IN THE OLIGOMYCIN SENSITIVITY OF THE MITOCHONDRIAL ATPase

#### Introduction

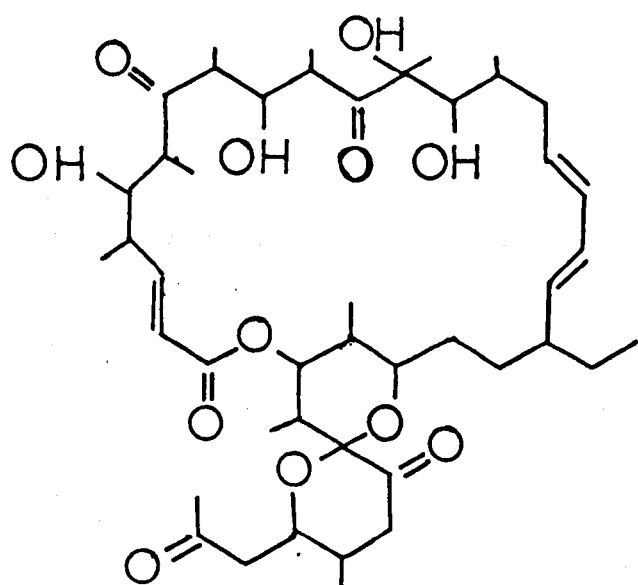
#### 7.1. Sterols and Respiratory Competency in Yeast

A link between the yeast sterol, ergosterol, and respiratory competence in yeast has been demonstrated by various authors. Parks and Starr<sup>103</sup> showed that there was a large increase in ergosterol biosynthesis when an anaerobic yeast culture was aerated, this increase being accompanied by the development of respiratory competence in the yeast cells. Kováč et al<sup>104</sup> have also analysed the changes in various lipid fractions from whole yeast cells that occur upon aeration of anaerobic cultures. They demonstrated that the rise in the respiratory quotient of the yeast cells was coincident with an increase in the cellular sterol content. However, Kováč et al<sup>104</sup> concluded that there was no empirical relationship between the lipid content of the yeast cells and their respiratory competence. Recently Thompson and Parks<sup>105</sup> have prepared a soluble cytochrome oxidase fraction from yeast which has ergosterol associated with the enzyme activity when solubilized from the membrane. It appears, therefore, that there is an intimate association between the yeast respiratory chain and the lipid mitochondrial membrane.

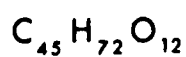
Kawaguchi<sup>106</sup> has examined the control of ergosterol biosynthesis showing that, as in cholesterol biosynthesis, there is a repressible step at the formation of mevalonic acid from 3-hydroxy-3 methyl glutaryl CoA. The inhibitors at this stage



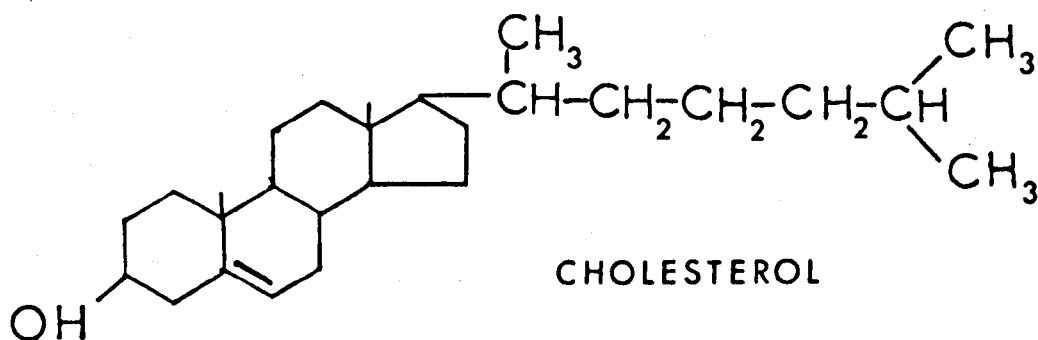
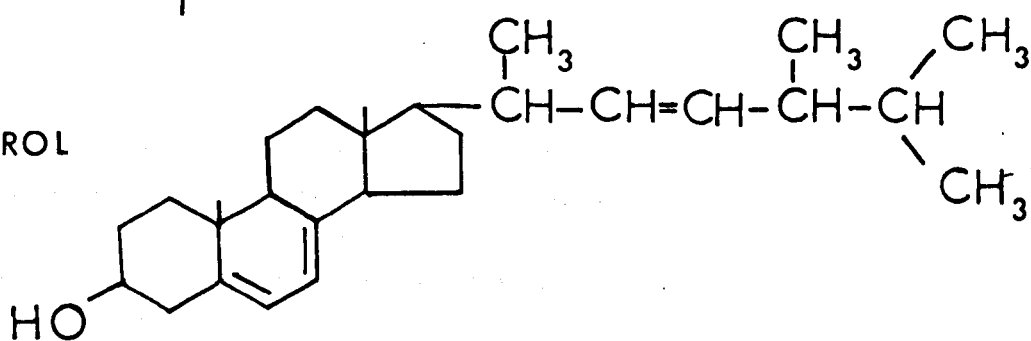
FILIPIN



OLIGOMYCIN B



ERGOSTEROL



CHOLESTEROL

Fig 7.1

were termed 'acidic lipids' derived from normal ergosterol catabolism.

## 7.2. Yeast Mutants and Sterol Metabolism

Resnick and Mortimer<sup>107</sup> have isolated various *S.cerevisiae* mutants which require oleic acid or ergosterol for growth. They found that there was a high coincidence between mutants having petite characteristics and which also require oleic acid or ergosterol for growth.

One of the non-petite mutants which required oleic acid for growth has been the subject of extensive investigation. This is the mutant KO-115 which has since been shown to be a nuclear mutant in the  $\Delta^9$  fatty acid desaturase enzyme, making the yeast incapable of converting palmitate to palmitoleate or stearate to oleate.<sup>108</sup>

Gordon et al,<sup>109</sup> have used this mutant to investigate the role of unsaturated fatty acids during the induction of respiratory function on aeration of anaerobic yeast culture. They found that both cytoplasmic and mitochondrial protein synthesis could be stimulated in KO-115 by adding unsaturated fatty acids after aeration of the culture. Proudlock et al<sup>110</sup> had previously investigated the uncoupling of oxidative phosphorylation in KO-115 and concluded that this uncoupling was not primarily due to an interference in protein synthesis, but was because of the lack of unsaturated fatty acids in the mitochondrial membrane.<sup>111</sup>

Another *S.cerevisiae* mutant originally isolated by Woods and Ahmed<sup>112</sup> has revealed a possible role for sterols in the action of polyene antibiotics on yeast cell membranes. The mutant, nys-3, was originally isolated because of its resistance to nystatin, a polyene

antibiotic which alters the ionic selectivity of the yeast cell membrane.<sup>113</sup>

The sterol content of the nys-3 cells has been shown to be radically altered, ergosterol having been replaced by a 28-carbon zymosterol-like sterol.<sup>114</sup> This new sterol has recently been identified as  $\Delta^{8(9),22}$  - ergostadiene-3  $\beta$ -ol which is observed transiently in normal aerobically adapting yeast.<sup>115</sup> The alteration of the sterol pattern complements an earlier observation that when ergosterol is added together with nystatin to a yeast culture the cells are no longer affected by nystatin,<sup>116</sup> implying that the free ergosterol is complexing with the nystatin and thus protecting the yeast cells from inhibition.

As well as being resistant to nystatin nys-3 was also found to be resistant to filipin, a smaller polyene macrolide antibiotic. Filipin has been shown to form stable adducts with cholesterol in an aqueous environment.<sup>117,118</sup> With both artificial and natural biological membranes the action of filipin is to change both the permeability and physical properties of the membranes.

It is apparent, therefore, that both these polyene macrolide antibiotics interact with sterols in biological membranes. Oligomycin, whose structure is shown in Figure 7.1, is also a macrolide antibiotic but unlike nystatin and filipin it is not a polyene since it does not have adjacent unsaturated carbon-carbon bonds. Thus these observations on filipin and nystatin may not apply directly to oligomycin but they do give an indication of the vital role of sterols in transmitting the effect of membrane modifying agents.

Recently Swanljung et al<sup>11</sup> have published results on the oligomycin resistant mutants of S.cerevisiae 022. The authors

show that at both the whole cell level and in a soluble OS ATPase preparation there was significantly more ergosterol present in the more oligomycin sensitive strain D22 WT than in the mutant strains D22 A16 and D22 A21. The conclusion was drawn that these mutants were resistant to oligomycin by virtue of a change in the hydrophobic nature of the mitochondrial membrane because of the altered ergosterol content of the cells. The significance of this report will be discussed later in relation to the results presented in this Chapter.

## Results

### 7.3. Ergosterol Content of the Yeast SMP

Although Swanljung et al<sup>11</sup> gave values for the ergosterol content of whole yeast cells and an OS ATPase complex no results were presented as to the ergosterol content of the yeast SMP.

Other workers at Warwick University had been unable to detect any difference in the ergosterol content of either whole yeast cells or the SMP derived from these cells, (M. Rhodes and K. Watson, personal communication). In order to resolve these apparent inconsistencies it was decided to measure the ergosterol concentrations of both yeast SMP and of the OS ATPase, described in the previous Chapter, using both D22 WT and mutant yeast strains.

These measurements were made using a GLC technique after the samples had been saponified and then extracted with petroleum ether, as described in Chapter 2, sections 2-17.

Processing the yeast SMP in this manner gave one main peak on the GLC trace which was identified as ergosterol by mass spectroscopy. There was no difference in the GLC traces using samples from D22 WT, D22 A12, D22 A16 and D22 A21 SMP. The preparation was

TABLE 7.1.

RELATIVE ERGOSTEROL CONTENT OF YEAST SMP MEMBRANES

The yeast SMP were saponified and the GLC performed on the extracts as described in Chapter 2, section 2.17. The saponifications were performed using S12 derived from two separate cultures for each yeast strain. The areas of the ergosterol peaks in the GLC traces are expressed relative to the area of the D22 WT ergosterol peak. The ratios shown are the averages for the two series of saponifications. In the second series of saponifications internal ergosterol standards were included and using these a value of 20.5  $\mu\text{g}$  ergosterol/mg S12 protein was calculated for the D22 WT SMP membranes.

Yeast Strain	Average ratio of areas corresponding to ergosterol peak in GLC trace.
D22 WT	1.0
D22 A12	1.5
D22 A16	0.95
D22 A21	1.55



duplicated, using SMP derived from different cultures of the yeast strains. The areas of the ergosterol peak in the GLC trace were measured and the ratios of these peaks relative to D22 WT peak were calculated. The figures shown in Table 7.1. represent an average value of the ratios obtained for each mutant from the two separate saponifications. The second series of saponifications also included internal ergosterol standards and using these standards the ergosterol content of D22 WT SMP was calculated to be 20.5  $\mu\text{g}$  ergosterol/mg protein. This compares with the value of 26  $\mu\text{g}$  ergosterol/mg protein given by Thompson and Parks<sup>105</sup> for freeze-thawed yeast mitochondria.

These results confirm those obtained at Warwick University that, compared to the parental D22 WT strain, there is no consistent alteration in the ergosterol content of the SMP from D22 A12, D22 A16 or D22 A21 strains, *that appears to be linked to oligomycin resistance.*

#### 7.4. Saponification of the OS ATPase

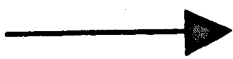
When the OS ATPase preparations described in the previous Chapter were saponified and analysed using the GLC technique various significant differences were observed between the D22 WT and mutant strains. The GLC traces of the saponified OS ATPase extracts are shown in Figures 7.2. and 7.3. As can be seen there is a complex pattern of peaks for all the samples, but that there was obviously more material present in the D22 WT OS ATPase extract than in the OS ATPase extracts from either the D22 A12, D22 A16 or D22 A21 strains.

It can be seen that the D22 A12 OS ATPase GLC trace, Fig. 7.2, is distinct from the GLC traces of the D22 WT OS ATPase saponification and those from the D22 A16 and D22 A21 OS ATPase saponifications. However, the GLC traces from D22 A16 and D22 A21 OS ATPases are very similar. This last observation is significant, since the SMP ATPase activities

FIGURES 7.2. AND 7.3.  
GLC TRACES OF SAPONIFIED EXTRACTS FROM  
OS ATPase PREPARATIONS

The saponification, extraction and GLC were performed as described in Chapter 2, section 2.17. The figures are to the same arbitrary scale for all the strains. The quantities of OS ATPase preparations that were saponified were, D22 WT, 0.16 mg., D22 A12, 0.12 mg., D22 A16, 0.08mg., and D22 A21, 0.1 mg.

Ionization in  
flame detector



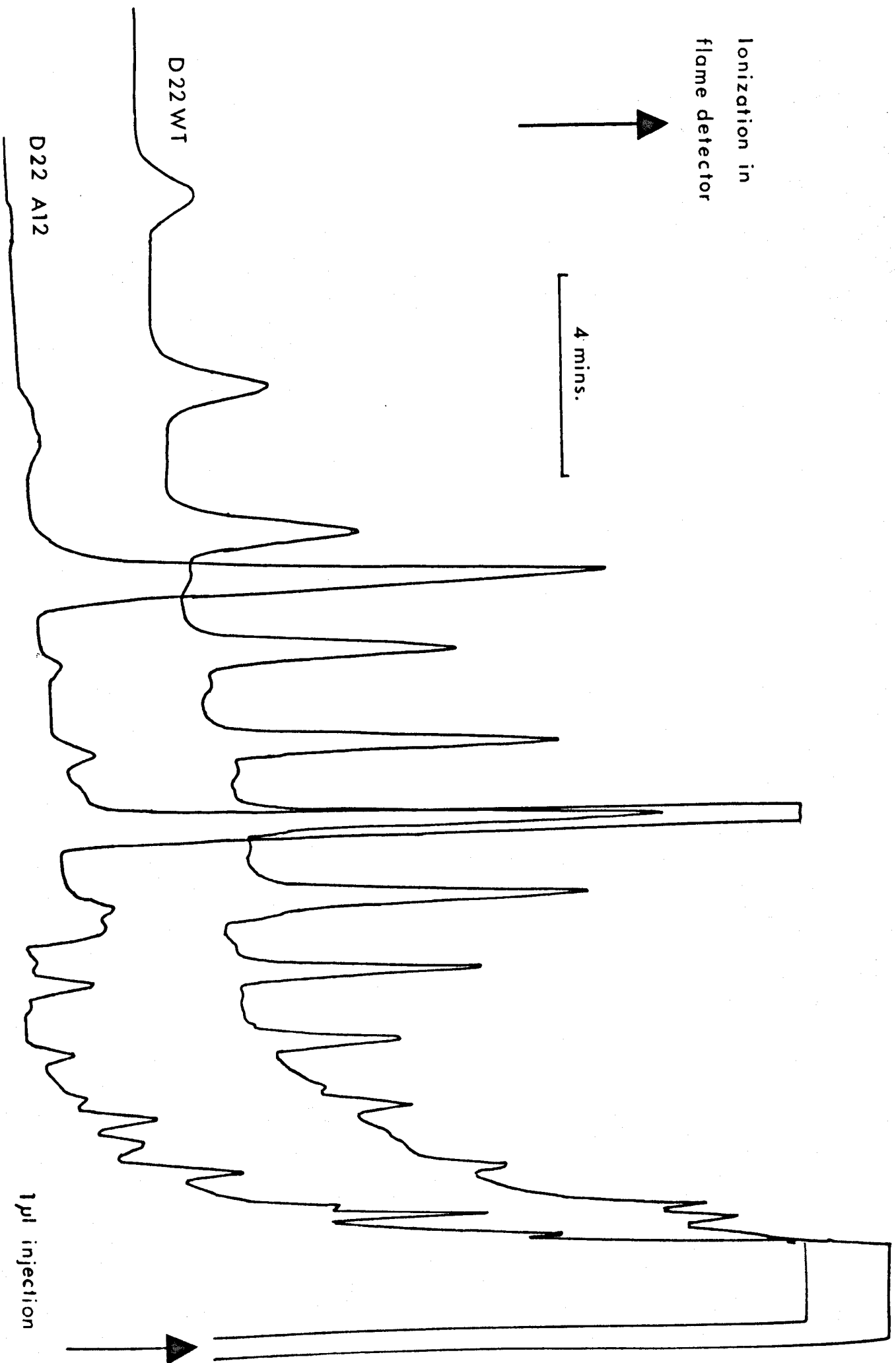
4 mins.



D22 WT

D22 A12

1  $\mu$ l injection



Ionization in  
flame detector

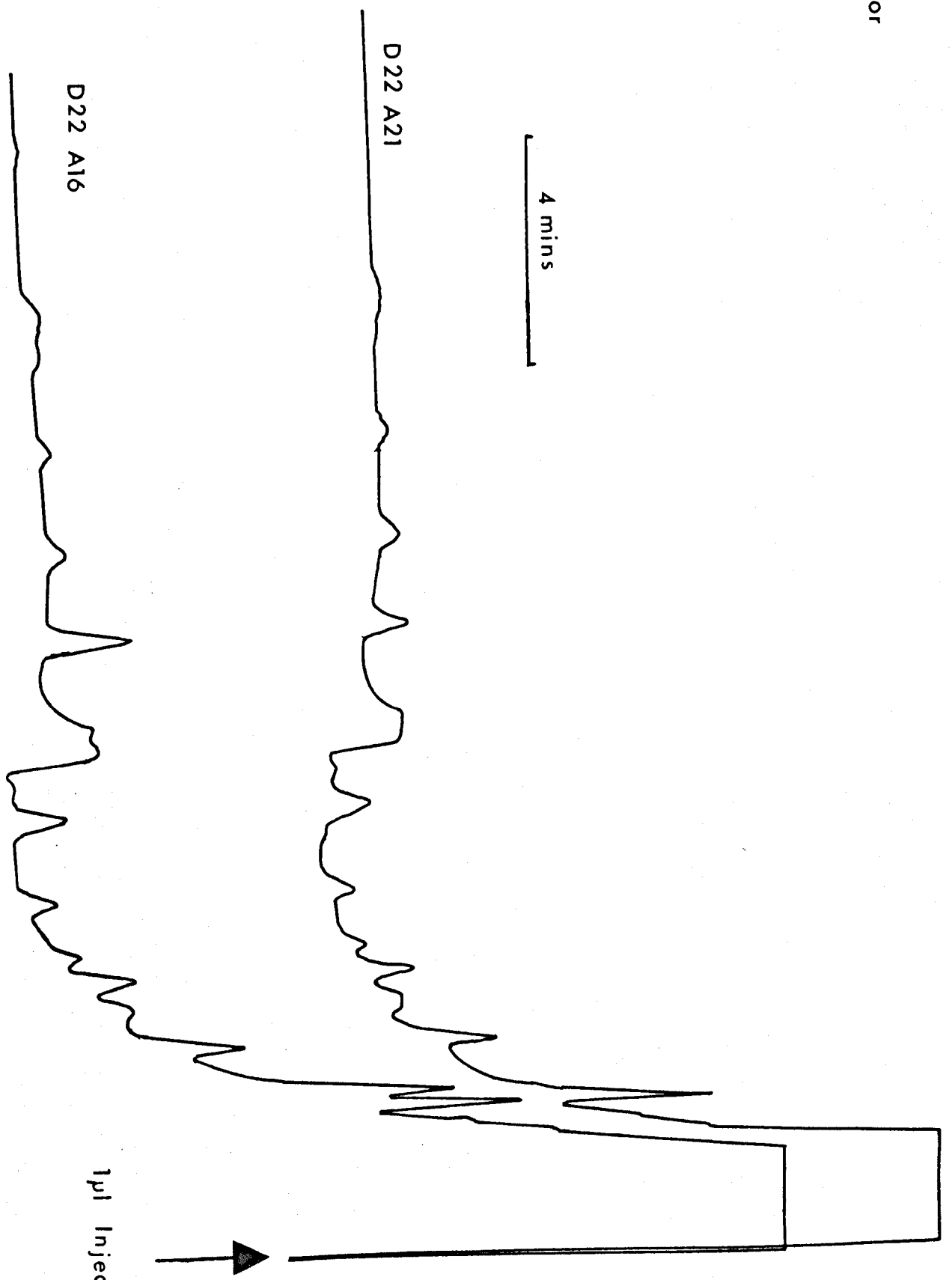


4 mins

D 22 A21

D 22 A16

1µl Injection



of these two mutants have identical oligomycin sensitivities, and also the behaviour of the OS ATPase complexes from D22 A16 and D22 A21 is very similar, as was shown in the previous Chapter. Recently it has been shown that D22 A16 and D22 A21 are allelic mutants, (P. R. Avner to be published).

The observation that the saponified extract from the D22 WT OS ATPase contains more material, as shown by GLC analysis, than similar extracts from D22 A12, D22 A16 and D22 A21 preparations has been repeated using OS ATPase complexes prepared from other cultures of the D22 WT and mutant yeast strains.

Mass spectroscopy was used in an attempt to identify some of the peaks on the GLC traces, but the mass spectra obtained were complex and not easily interpreted. Various preliminary observations were made using this technique and these are given below:

(1) No ergosterol could be detected in the GLC traces from the OS ATPase preparations, unlike the GLC elution profile of the saponified SMP extract.

(2) From the pattern of peaks obtained in the GLC trace of the D22 WT OS ATPase preparation it was suggested that these peaks may represent a series of compounds with long hydrocarbon side chains.

(3) The mass spectra of several of these peaks on the D22 WT GLC trace supports the idea of long hydrocarbon chains being involved in the compounds.

(4) The two prominent peaks in the D22 A12 GLC elution profile also give a hydrocarbon chain breakdown pattern when examined by mass spectroscopy.

(5) The largest mass fragments detectable in the two major peaks on the D22 A12 GLC trace both had a M.W. of over 350.

### 7.5. Selective Labelling of yeast mitochondrial lipids

Since it was apparent that a lipid fraction in the OS ATPase preparation was significantly different in the mutant yeast an attempt was made to selectively label the mitochondrial lipids as a possible means of analysing differences in the D22 WT and mutant mitochondrial membranes.

Initially D22 WT cells were grown in the presence of ( $^{14}\text{C}$ ) - mevalonic acid, an intermediate in ergosterol biosynthesis, but only a negligible amount of radioactivity ( $<0.02\%$  total radioactivity) was found to be associated with the SMP. This confirms an earlier report that the uptake of mevalonate by growing yeast cells is very poor.<sup>119</sup> Another approach tried was the growth of cells in the presence of ( $^{14}\text{C}$  - methyl) methionine, since adenosyl methionine acts as a methyl donor during ergosterol biosynthesis.<sup>120</sup> 64% of the ( $^{14}\text{C}$ ) - L - methionine in the culture medium was incorporated into the cells, 4% of the cellular radioactivity being associated with the SMP membranes. However, 85% of the SMP radioactivity was associated with protein and not lipids. To achieve a selective labelling of the yeast lipids in the absence of protein labelling it will probably be necessary to perform anaerobic to aerobic shifts in yeast cultures inhibited in the presence of erythromycin,<sup>121</sup> using ( $^{14}\text{C}$  methyl) - methionine as a labelling agent.

### 7.6. Solvent extractions of yeast SMP

In order to further study the role of lipids in determining the oligomycin sensitivity of the SMP ATPase activity various solvent extractions were performed on the yeast SMP. Järnefelt<sup>122</sup> has shown that extraction of a membrane-bound  $\text{Na}^+/\text{K}^+$  ATPase with hexane removed almost all of the membrane cholesterol, but did not affect

the phospholipid content of the membranes. The ATPase activity was unaffected by this extraction. This treatment was applied to yeast SMP but neither the ATPase activity nor the oligomycin sensitivity of this activity were affected by the hexane extraction.

Drabikowski et al<sup>123</sup> have used solvent extraction techniques to investigate the  $\text{Ca}^{++}$  ATPase activity bound to the sarcoplasmic reticulum membrane vesicles. Mild treatment of the membrane vesicles with aqueous diethyl ether lead to a partial loss of the membrane cholesterol, and the vesicles could no longer accumulate  $\text{Ca}^{++}$ . However, if the vesicles were lyophilized prior to the extraction and this extraction was performed using dry diethyl ether all the membrane cholesterol was extracted, but the ability of the vesicles to accumulate  $\text{Ca}^{++}$  was not lost. Very little phospholipid was extracted with either treatment. The authors<sup>123</sup> concluded that the loss of ability to accumulate  $\text{Ca}^{++}$  was primarily due to the action of diethyl ether on the membranes, rather than a loss of cholesterol.

Using the procedure described in the legend to Figure 7.6 a diethyl ether extraction was performed on yeast SMP that had been prepared from commercial baker's yeast cake by the liquid  $\text{N}_2$  technique of Tzagoloff.<sup>89</sup> Prior to the diethyl extraction the yeast SMP had an ATPase specific activity of  $0.4 \mu\text{moles}/\text{min}/\text{mg. protein}$  which was 50% inhibited by  $6.0 \mu\text{g oligomycin}/\text{mg. SMP protein}$ . After the ether extraction the ATPase specific activity was slightly stimulated to  $0.5 \mu\text{moles}/\text{min}/\text{mg.}$  However, it was found that the ATPase activity in the ether-extracted particles could only be 10% inhibited by  $50 \mu\text{g oligomycin}/\text{mg. SMP protein}$ . When the ether extract

was examined using a TLC system suitable for separating neutral lipids all that could be detected were compounds running with the same Rf values as free and esterified ergosterol. Spraying with the phosphomolybdenum blue reagent gave a faint blue colour at the base line of the TLC plate, indicating the presence of some phospholipid in the extract.

No quantitative information is available at present on either the percentage of SMP membrane ergosterol that is extracted by this procedure or the quantity of phospholipid present in the ether extract. Experiments are being conducted at present to determine these values, (R. B. Beechey, personal communication).

After ether extraction it was found that the yeast SMP ATPase activity was much less stable than the native SMP. After being thawed from storage at  $-20^{\circ}\text{C}$  the ATPase specific activity declined from 0.4  $\mu\text{moles/min/mg}$  to 0.15  $\mu\text{moles/min/mg}$  when kept at  $+4^{\circ}\text{C}$  for 12 hours. The ATPase activity of untreated SMP is quite stable over this period when kept at  $+4^{\circ}\text{C}$ .

A series of additional experiments were performed in an attempt to determine whether the ATPase oligomycin sensitivity had been lost due to SMP membranes being damaged directly by the diethyl ether treatment, or whether the removal of one of the membrane lipid fractions from the SMP was alone responsible for the loss of oligomycin sensitivity. The results of these experiments are shown in Figure 7.4. The sterols or the concentrated ether extract were added to the ether extracted SMP and then incubated at  $30^{\circ}\text{C}$  for 2 hours. After this incubation the % inhibition of the ATPase activity caused by 50  $\mu\text{g}$  oligomycin/mg SMP protein was



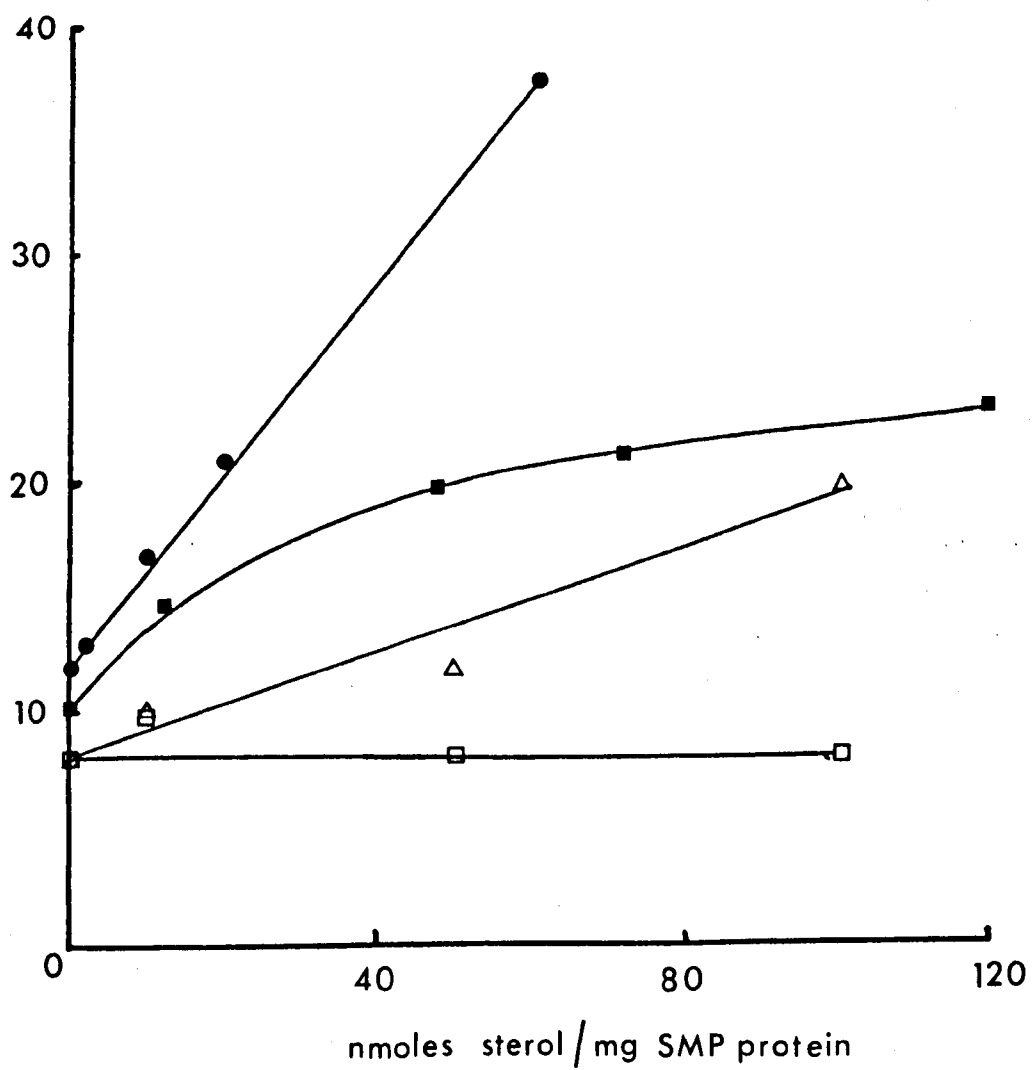
FIGURE 7.4.

STEROL RECONSTITUTION EXPERIMENTS WITH  
ETHER EXTRACTED YEAST SAT

Ether extracted yeast SAT, as described in Chapter 2, section 2.16, were incubated with the sterols at the indicated concentrations for 2 hours at 30°C. The sterols were dissolved in diethyl ether and then added to the ether extracted yeast SAT which were at a protein concentration of 30 mg/ml in 0.25 M sucrose, 0.1 M Tris pH 7.5. The ATPase activity was measured at pH 9.5 and 30°C, and then the inhibition caused by adding 50 µg oligomycin/mg protein to each incubation was determined. This % inhibition of the absolute ATPase activity is shown for each sterol that was used.

- Ether extract
- Ergosterol
- △ Cholesteryl stearate
- Cholesterol.

% Inhibition of ATPase  
by 50  $\mu\text{g}$  oligomycin/mg protein



determined.

Figure 7.4 shows that the ether extract was the most effective agent in partially restoring oligomycin sensitivity to the ATPase. Ergosterol had some effect, cholesteryl stearate had a marginal effect at high concentrations, but cholesterol had no effect in restoring oligomycin sensitivity.

Further work using the diethyl ether extraction technique showed that the yeast SMP also lose their sensitivity to DCCD on ether extraction. DCCD at concentrations up to 20 nmoles DCCD/mg SMP protein had no effect when it was incubated with yeast SMP that had been extracted with diethyl ether. The SMP ATPase activity from the commercial bakers yeast was normally 50% inhibited by 1.7 nmoles DCCD/mg SMP protein, after incubation at 30°C for 1 hour.

#### 7.7. Discussion and Conclusions

The results presented here are a preliminary study, but they do indicate the importance of lipids in the ATPase complex, especially with regard to the oligomycin sensitivity of the ATPase.

The saponification/GLC studies have demonstrated, in agreement with other studies, that there is no significant difference in the ergosterol content of SMP derived from D22 WT, D22 A12, D22 A16 or D22 A21 strains. The results do not agree with the hypothesis of Swanljung et al<sup>11</sup> that there is a lower ergosterol content in the oligomycin resistant yeast strains, and that this lower ergosterol concentration in the ATPase complex is linked to the oligomycin resistance seen in the yeast mutants. It was stated<sup>11</sup> that growth of the mutants in the presence of ergosterol had no effect on the mitochondrial ergosterol content, nor on the oligomycin sensitivity of the ATPase

complex, (P. Swanljung, personal communication). These observations would suggest that the mutants cannot be deficient in their ability to synthesize ergosterol. An alternative explanation for the observations of Swanljung et al<sup>11</sup> is that an alteration in the yeast cell membranes causes a lower amount of ergosterol to associate with the membrane proteins.

However, with the reported five fold decrease in the cellular ergosterol content of the mutant strains one would have expected other profound changes to occur in the cellular metabolism, but so far both growth rate and growth yield studies have failed to reveal any such difference between the WT and mutant strains.<sup>80</sup>

The GLC analyses of the CS ATPase preparations have disclosed several important differences between D22 WT and the mutant strains studied so far.

The most obvious is that in this saponified extract there is far more material from the D22 WT CS ATPase preparation than from the mutant strains D22 A12, D22 A16 and D22 A21.

The two allelic mutants, D22 A16 and D22 A21 show almost identical GLC traces, indicating that the differences seen between these mutants and D22 WT may well be closely linked to oligomycin resistance.

The differences in the GLC traces appear to be relative rather than absolute, i.e., there are still peaks on the mutant GLC traces which correspond to the major peaks seen on the D22 WT GLC trace.

It is not felt that the large number of peaks seen in all the GLC traces is artefactual, since these peaks were not seen when either ergosterol or yeast ATP were put through the same process

of saponification and extraction.

The identification of these peaks can only be conjectural at present, but the evidence so far suggests the involvement of long hydrocarbon side chains in the saponified extracts.

The diethyl ether extraction procedure described in this Chapter offers scope for further work to investigate the role of sterols in the SMP membrane, especially with regard to the oligomycin sensitivity of the ATPase complex. This ether extraction may cause loss of oligomycin sensitivity by extracting <sup>c</sup> necessary lipid components or merely by damaging the membrane, as was suggested by Drabikowski et al<sup>123</sup> in the case of diethyl ether extraction of a  $\text{Ca}^{++}$  ATPase.

The addition experiments do indicate that the loss of oligomycin sensitivity is at last partially reversible, although the same degree of oligomycin sensitivity found in the yeast SMP prior to ether extraction was never achieved. This would indicate that the present experimental technique may be damaging the yeast SMP membrane and an examination of other extraction techniques such as the use of lyophilized SMP may well solve this problem. The specificity of the sterols in reconferring oligomycin sensitivity is significant since cholesterol, which does not occur in yeast mitochondrial membranes, has no effect, whereas the normal yeast sterol, ergosterol, does partially add back oligomycin sensitivity.

Analysis of the ether extract by TLC showed the presence of esterified as well as free ergosterol, plus some phospholipid, so that a combination of all three lipid types may be necessary to obtain a maximal reconferral of oligomycin sensitivity.

The role of sterols in the ATPase oligomycin sensitivity

may either be to hold the SMP in the correct configuration so that oligomycin can inhibit the ATPase complex, or possibly to provide a hydrophobic sink in which the oligomycin can collect and thus inhibit the ATPase complex.

The selectivity of ergosterol compared to cholesterol in reconferring oligomycin sensitivity suggests that the former is correct. Also the fact that after diethyl ether treatment the SMP ATPase activity is stimulated suggests a degree of membrane rearrangement. The observation that ether extracted yeast SMP are far less stable, as shown by the decay in ATPase activity, than the normal SMP suggests that sterols may have an important role as an agent for maintaining the correct membrane conformation.

## CHAPTER 8

### CONCLUSIONS

The results presented in this thesis are those obtained during an investigation into the biochemistry of various oligomycin resistant mutants of S.cerevisiae D22.

Two of the methods used in this investigation were designed to study the mode of oligomycin inhibition of yeast SMP ATPase activity and as a consequence to give some idea of the mode of oligomycin resistance in the yeast mutants. The first of these methods, the differential  $^{32}\text{P}$ i incorporation experiments using aurovertin and oligomycin did not prove successful either in elucidating the mode of oligomycin inhibition or in studying the yeast mutants. Various suggestions were made as to the cause of this failure to repeat previous work<sup>6,7</sup> but no firm conclusions could be drawn as to why the differential  $^{32}\text{P}$ i incorporation was not seen. The results do suggest that perhaps the present interpretation on the relative sites of oligomycin and aurovertin inhibition should be reviewed, especially since the previous evidence provided by the effect of aurovertin and oligomycin on arsenate-stimulated respiration in mitochondria has recently been shown to be uncertain.

The approach of using DCCD was next used, but this was not successful as a means of investigating the biochemistry of the mutants. It had been hoped that DCCD would bind covalently to yeast SMP and thus it would be possible to isolate a DCCD-binding protein, as has been done in beef heart mitochondria.<sup>9</sup> However, it was shown that DCCD did not bind to yeast SMP, as it does to

beef heart mitochondria. The results do provide evidence that the DCCD was inhibiting the yeast SMP ATPase activity by acting as a condensing agent, promoting the formation of an intramembrane bond. The proposed mechanism is very similar to that given for the inhibition of red blood cell ATPase activity by EDAC.<sup>79</sup> Although DCCD was shown not to bind to yeast SMP and thus was not suitable for the investigation originally proposed the results do show that radiolabelled nucleophiles such as GEE might well be suitable for investigating the site of DCCD action on yeast SMP membranes. The accessibility of the added nucleophiles might well give an insight into the changes that have occurred on the yeast SMP membrane.

An alternative method used for investigating the biochemistry of the oligomycin resistant yeast mutants, was the comparative analyses of the SMP structure, trying to correlate any changes that may have occurred in the SMP composition with the strain-dependant oligomycin resistance seen in the yeast.

No gross changes were seen in the SMP protein composition, as measured by SDS polyacrylamide gel electrophoresis. Similarly no difference could be found in the lipid content of the yeast SMP, except that the mutants D22 A16 and D22 A21 appeared to have less fatty acid associated with the SMP membrane than the D22 WT strain. These results do not support the hypothesis of Swanljung *et al*<sup>11</sup> that the ergosterol content of the yeast mutants is significantly lower than the D22 WT strain and that this lower ergosterol content is linked to the oligomycin resistance of the mutants. No such difference was found in the SMP ergosterol concentrations, supporting earlier observations (N. Rhodes, personal communication).



As discussed earlier a change such as the five fold decrease in ergosterol concentration<sup>11</sup> would almost certainly have had other metabolic consequences than an alteration of ATPase oligomycin sensitivity. No changes have been observed in the metabolism of the yeast mutants, apart from their resistance to oligomycin.

The analytical approach was further refined by preparing a soluble oligomycin sensitive ATPase preparation from yeast SMP. Again no differences were detected in the protein composition of the CS ATPase preparations from D22 WT, D22 A12, D22 A16 and D22 A21. The free fatty acid content of these CS ATPase complexes did not appear to be significantly different when examined by TLC. Possibly, however, there may be more phospholipid associated with the D22 WT CS ATPase than the D22 A16 and D22 A21 preparations.

Saponified extracts from the CS ATPase preparations did show a significant strain dependant difference between the D22 WT and mutant strains. There appeared to be much more lipid material associated with the D22 WT CS ATPase preparation when measured by this method. There was a significant correlation between the strain and the resulting GLC traces, D22 A16 and D22 A21, allelic mutants, have very similar GLC traces which are different from both the D22 WT and another mutant, D22 A12. The identification of these various fractions seen in the GLC trace can only be speculative at the moment, but the fractions appear to be high MW compounds with long hydrocarbon chains present. The results suggest a type of lipid, possibly fatty acids. An alteration in the local lipid environment around the ATPase complex within the SMP membrane may well explain the oligomycin resistance seen in the mutant yeast,

a subtle change in the internal fluidity of the SMP membrane may well render the ATPase activity less likely to respond to the presence of oligomycin. Alternatively a lowering of the hydrophobic environment around the ATPase complex may lessen the probability of oligomycin reaching the site at which it inhibits the ATPase activity.

The D22 WT strain does appear to be more susceptible to changes within the local environment around the ATPase complex, as shown by the greater variation in the SMP ATPase oligomycin sensitivity in D22 WT compared to the mutant SMP. This instability is more marked in the D22 WT strain when the soluble CS ATPase preparations from WT and mutant are compared. Unlike the mutant strains D22 WT loses both ATPase activity and the oligomycin sensitivity of this activity when the ATPase complex is removed from the SMP membrane. It appears, therefore, that the mutation to oligomycin resistance has, as a consequence, altered the stability of the SMP ATPase complex as well. This could be accounted for by a change in the lipid environment that is associated with the ATPase complex. The reason for this change may be a biosynthetic deficiency in the mutants, or a change in the ATPase complex so that less lipid is associated with this within the membranes of the oligomycin resistant mutants. At present these two possibilities cannot be distinguished.

D22 A16 and D22 A21, although allelic and having identical oligomycin sensitivities for the SMP ATPase activity, are not equally sensitive to DCCD. The SMP ATPase activity from D22 A21 is much more resistant to DCCD than the D22 A16 SMP ATPase. This observation cannot be explained at present except that perhaps subtle changes in

the SMP membrane have more acute effects on the DCCD sensitivity than the oligomycin sensitivity.

The ether extraction procedure described in the last Chapter also illustrates the importance of lipids in the ATPase complex, particularly with respect to the oligomycin sensitivity of the ATPase. Further work is necessary to optimise both the ether extraction technique and the readition of sterols to the extracted SMP. The use of lyophilized SMP for extraction and sonicating the ether extracted SMP in the presence of the added sterols are two obvious techniques that may give a higher degree of oligomycin sensitivity in the reconstituted particles. The observation that ether extracted SMP are much less stable, as judged by ATPase activity, than the native SMP also indicates the vital role of lipids, and sterols in particular, in maintaining the SMP membrane structure.

The sensitivity<sup>to oligomycin</sup> of mitochondrial ATPase activity from other yeast strains is given in Table 8.1. This shows the mitochondrial ATPase activity from other yeast strains that have been used in research is far less sensitive to oligomycin than the S.cerevisiae D22 WT strain. One of the strains, S.cerevisiae D261, has an ATPase activity which is more resistant to oligomycin than the most resistant mutants, D22 A16 and D22 A21, that were used in this study. This observation does not invalidate the work but it indicates the possibility that D22 WT is itself a mutant which is hypersensitive to oligomycin and the resulting oligomycin resistant mutants from D22 WT may well be partial revertants to a true WT phenotype. This would explain the anomalous behaviour of soluble OS ATPase preparation from D22 WT which loses its oligomycin

TABLE S.1.

OLIGOMYCIN SENSITIVITY OF MITOCHONDRIAL

ATPASE ACTIVITY FROM YEAST STRAINS

Yeast Strain	Method of preparing mitochondria	Oligomycin sensitivity of ATPase activity ( $\mu$ g oligomycin/mg protein)	Reference
<u>S. cerevisiae</u> D261	Braun mitochondria (Glass bead disruption)	50% inhibition at 43.0*	125
<u>S. cerevisiae</u> D261	Snail-gut mitochondria (Enzymic digestion of cell wall)	30% inhibition at 30.0	126
<u>S. cerevisiae</u> D273-10B	Braun mitochondria	50% inhibition at 17.5	127
'Diploid' <u>S. cerevisiae</u>	Liquid nitrogen mitochondria (Frozen yeast disrupted in a Waring blender)	50% inhibition at 10.0	84
<u>S. cerevisiae</u> 1020 and 706	Snail-gut mitochondria	90% inhibition at 330.0*	4
'Bakers Yeast'	Brownhill shaker mitochondria (Glass bead disruption)	50% inhibition at 9.0	81
<u>S. cerevisiae</u> KD-115	Snail-gut mitochondria	70% inhibition at 60.0	128
<u>S. cerevisiae</u> D22	Braun SM	50% inhibition at 1.5	Present Results
WT		50% inhibition at 7.0	
A12		50% inhibition at 22.0	
A16		50% inhibition at 22.0	
A21		50% inhibition at 22.0	

\* Calculated from figures in paper

sensitivity, unlike both strains used by Tzagoloff and Meagher,<sup>81</sup> and the oligomycin resistant strains of S.cerevisiae D22.

For future work one must consider whether the present oligomycin resistant mutants will be suitable for determining the role of the various factors present in the ATPase complex. The results in this thesis suggest that the mode of resistance in strains D22 A12, D22 A16 and D22 A21 is a fairly subtle change in the local lipid environment around the SMP ATPase complex. To detect the cause of this lipid alteration will probably prove very difficult, and to further demonstrate that this alteration is linked to oligomycin resistance will be even more difficult. It may prove necessary to isolate other yeast mutants which show a much higher degree of resistance to oligomycin in the SMP ATPase activity than those mutants which have been studied so far.

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A PAPER GIVEN AT THE BIOCHEMICAL  
SOCIETY MEETING IN NOTTINGHAM, DECEMBER 1972.

The effects of extraction with diethyl ether on the sensitivity  
to inhibitors of mitochondrial ATPase activity

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ABSTRACT

Extraction of submitochondrial particles from beef heart and Saccharomyces cerivisiae with diethyl ether results in the insensitivity of the submitochondrial particle bound ATPase activity to the inhibitors oligomycin, venturicidin and DCCD.

We have reported previously that the purified preparations of oligomycin-sensitive mitochondrial ATPase isolated from wild type strain D22 and oligomycin resistant strains (D22 A16 and D22 A21) of Saccharomyces cerivisiae do not appear to have major variations in the protein-subunit composition based on analyses by polyacrylamide gel electrophoresis. However there are major differences in the lipid components of the oligomycin-sensitive ATPase preparations isolated from the wild type and mutant strains (Broughall, Griffiths and Beechey, 1972). This apparent relationship between lipid composition and oligomycin-sensitivity led us to investigate the



effects of extraction with organic solvents on the oligomycin-sensitivity of the ATPase activity located on submitochondrial particles. These preliminary results show that the inhibitory effects of oligomycin (Lardy, Johnson and McMurray, 1958) venturicidin (Walter, Lardy and Johnson, 1967) and DCCD (Beechey et al, 1966) on the submitochondrial particle-bound ATPase activity cannot be manifested after the membrane has been extracted with diethyl ether.

Submitochondrial particles suspended in 0.25M-sucrose, 10mM-tris sulphate pH 7.5 (10mg of protein/ml) were extracted with an equal volume of peroxide-free redistilled diethyl ether, by mixing for 2 x 10 sec. periods with a vortex mixer. The resulting suspension was separated by centrifugation at 1000g for 4 min. at room temperature. The diethyl ether was removed by aspiration and the submitochondrial particles were resuspended in the aqueous phase and stored in ice. The relative volumes of diethyl ether and submitochondrial particles suspension do not appear to be critical.

This extraction of either yeast or beef-heart submitochondrial particles with diethyl ether results in a change of the ATPase activity of the membrane. Often a slight stimulation is noted, but occasionally a decrease of activity as great as 50% is found. However, the remaining ATPase activity is almost totally insensitive to the inhibitors of ATP synthesis, DCCD, venturicidin and oligomycin at concentrations ten times greater than those which normally give 90% inhibition. The results of a typical experiment are shown in Table 1.

Table 1. Effect of oligomycin on the adenosine triphosphatase activity of control and ether-extracted submitochondrial particles isolated from ox heart mitochondria.

Preparation	Adenosine triphosphatase activity ( $\mu\text{mol}/\text{min}$ per mg of protein)
Control submitochondrial particles	0.42
□ + 15 $\mu\text{g}$ of oligomycin/mg of protein	0.05
Extracted submitochondrial particles	0.50
□ + 15 $\mu\text{g}$ of oligomycin/mg of protein	0.41

The oligomycin-insensitive ATPase activity of extracted submitochondrial particles declines on storage at 0°. This raised the possibility that the diethyl ether treatment releases the ATPase molecules from the membranes. Electron microscopic examination of negatively stained control and ether-extracted beef heart submitochondrial particles showed that there are relatively few inner membrane spheres (mitochondrial ATPase) in diethyl ether extracted membrane. Generally the appearance of the extracted membranes is less granular than those of the control submitochondrial particles. The extracted particles seem to lie flatter on the grid. However, the loss of the

oligomycin-sensitivity is not due to the dissociation of the ATPase molecules from the membrane. This was shown by extracting submitochondrial particles with diethyl ether. The diethyl ether layer was removed and the submitochondrial particles sedimented by centrifugation. The aqueous supernatant contained no ATPase activity, whilst the submitochondrial particle preparation retains the ATPase activity. Also, polyacrylamide gel electrophoretic analysis of control and ether-extracted submitochondrial particles shows that there is neither an obvious loss of a component nor a change in the relative proportions of the various components in the gels.

The loss of oligomycin-sensitivity is not due to the physical presence of diethyl ether in the membrane, since reconstituted freeze-dried submitochondrial particles which have had all the diethyl ether removed by freeze drying have an ATPase activity which is oligomycin insensitive.

It is not probable that diethyl ether treatment removes the site of action of these inhibitors from the membrane. Knight et al (1968) have shown that this site is a proteolipid and further (unpublished data) results have shown that this molecule is not extracted by diethyl ether from beef heart mitochondria. However, in agreement with the findings of Lenez et al (1972) we noted that diethyl ether extractions removed approximately 14% of the total submitochondrial particle phosphate content, thus indicating that the extraction procedure has a certain degree of selectivity. This presumably is based on the accessibility of the membrane phospholipids to diethyl ether.

The mode of action of diethyl ether in decreasing the sensitivities of membrane-bound ATPase to oligomycin etc. can either be caused by a major disruption and subsequent rearrangement of the membrane, or by diethyl ether removing a membrane constituent, the presence of which is necessary for the inhibitors DCCD, venturicidin and oligomycin to reach the site of action.

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